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SUBCELLULAR PARTICLES IN THE NEOPLASTIC PROCESS

BY

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Part I. General Considerations

CLONAL DERIVATION OF VIRUSES

By R. Dulbecco

California Institute of Technology, Pasadena, California

The general problem of the clonation of animal viruses can be separated into three different elements: (1) the existence of virus clones; (2) the methods of clonation; and (3) the genetic purity of the clones. I propose to examine these three elements briefly in the light of the experience that my associates and I have gained from our experimental work.

The Existence of Virus Clones

Clones exist if a cell can be infected by a single virus particle. Evidence demonstrating that a single virus particle is sufficient to infect a cell has been recently presented (Dulbecco and Vogt, 1955). The evidence is based on two arguments of a statistical nature: first, that concerning the existence of a linear relationship between the concentration of the virus and the number of plaques (Dulbecco, 1952; Dulbecco and Vogt, 1954) or pocks (Rubin, 1955) it produces; second, that dealing with the exponential inactivation of the plaque-forming ability of several viruses under the action of various agents, such as ultra-violet light, X rays, heat, and antibody.

More direct evidence in favor of the concept that the infection of a cell or a plaque can be initiated by a single virus particle may be obtained by performing a mixed-infection experiment with two related virus lines. Experiments of this nature were carried out by using two lines of poliomyelitis virus type 1, differing in a single genetic character: the sensitivity to inactivation by heat (Dulbecco and Vogt, 1956). The two lines are designated as t^s (temperature sensitive) and t^r (temperature resistant), respectively. The experiments were performed by infecting cells with a mixture of the two viruses. The multiplicity of infection—that is, the average number of virus particles attached to each cell—for each of the two lines could be calculated in each experiment from the plaque titer of the virus lines used and from their rate of attachment to the cells. The multiplicity of the t^r virus was always rather high, between 5 and 15 per cell; the multiplicity of the t^s virus was varied between 0.7 and 7. Thus, practically all the cells were infected by the t^r virus, but only a proportion of the cells, varying in different experiments, was infected by the t^s virus. The yield of single cells was collected by using a technique previously described (Lwoff *et al.*, 1955); the heat character of all individual particles of each yield was determined. Thus, the proportion of cells yielding either pure t^r virus or pure t^s virus, or a mixture, was measured in each experiment.

The results of these experiments can be summarized as follows: in every experiment the proportion of cells that was expected not to have received any t^s particle—from the multiplicity of the t^s virus—had a pure t^r yield.

Due to an exclusion produced by the t^s virus, the proportion of cells that was expected to be mixedly infected always yielded the t^s virus, but not always the t^r type.

These results support the hypothesis that the infection of a cell as well as a plaque are initiated by a single virus particle. The reasons are as follows:

If the attachment of many virus particles, say n , to the same cell was required for infection, then a plaque-forming unit would contain n or more particles. In the mixed-infection experiment, in which an excess of t^r virus was added to the t^s virus, the n particles necessary to start the infection of a cell could be provided by a collaboration of both viruses, since they differed only by one genetic character. Therefore, the n or more particles contained in a plaque-forming unit of the t^s virus would be distributed over more than one cell. Since the t^s particles have a marked advantage over the t^r particles, all these cells should yield the t^s virus. Thus, under this assumption, the proportion of cells yielding t^s virus should have been higher than the proportion calculated from the titer of the virus in p.f.u., although the experimental results did not bear out such an expectation.

These experiments afford, at the same time, a direct demonstration that virus clones, pure for one character, can be obtained from cells exposed to a mixture of two virus lines. In the reported experiments the cells yielding a pure t^r clone were infected by various numbers of particles identical for the t^r character. When virus populations of unknown composition are used, only cells infected by a single virus particle give assurance of yielding a pure virus line.

Methods of Clonation

I shall first examine this problem from a statistical standpoint. Let us consider an experiment in which a population of virus particles is mixed with a population of cells; after a proper time of interaction, this population contains a mixture of noninfected cells, cells infected by one virus particle, by two particles, and so on. Although the average number of virus particles infecting the cells is known accurately, the actual number infecting a given cell is unknown. Cells infected by a single virus particle can be isolated only by using conditions that make the class of cells infected by only one particle much larger than the class infected by more than one particle. This condition is obtained by mixing a small number of virus particles with a large number of cells. Even in this case, however, a fraction of the infected cells is infected by more than one particle; if m , the multiplicity of infection, is small, this fraction is equal to $m/2$, according to the Poisson distribution.

We can now examine the two known experimental methods for virus clonation: (1) the localized-lesion method and (2) the terminal-dilution method. In the localized-lesion method, one takes advantage of the existence of a localized virus population in the lesion. The lesion may be a plaque obtained in tissue culture, or a pock on the chorioallantoic membrane of the chicken embryo. In this method the number of cells of the culture or of the membrane is very large, of the order of 10^6 or more. The number

of virus particles that start infection is small; let us assume that it is equal to 100. Thus, $m = 10^{-4}$. Under these conditions practically all the infected cells are infected singly. The lesion, however, can be identified only when many cells show changes; two independently infected cells may give rise to a unique lesion. The value of m must therefore be referred, not to the individual cells, but to the groups of cells included in a recognizable lesion. If we assume that this number is at least 10^3 , m increases to 10^{-1} in our example; thus, the probability that a lesion is of multiple origin may not be negligible.

To improve the probability of finding a pure virus clone in a lesion, the number of lesions per culture or membrane must be minimal. If this number is unity— $m = 10^{-3}$ under the conditions assumed here—the method is then very satisfactory and, by repeated serial clonation, can be made as safe as desired.

In the terminal-dilution method, m is the average number of infectious particles per assay unit—that is, per tube culture or animal. If m is small, $m/2$ measures the probability that, in an infected tube, growth has started from more than one particle. If 1 out of 10 tubes shows virus development, the growth has started from more than 1 particle in 5 per cent of the infected tubes. Therefore, the chance of obtaining a pure clone by the terminal-dilution method becomes sufficiently large only in experiments involving the use of many assay units, the great majority of which must be noninfected.

The isolation of a pure clone does not depend solely on the fulfillment of the discussed statistical requirements, but on the fulfillment of stringent experimental conditions, as well. In this connection, two main points must be considered. First, in all methods considered above, only a fraction of the virus placed in contact with the cells becomes attached to them; the unattached virus contaminates the clone unless it is removed before the progeny virus is produced. The removal of the free virus can be carried out adequately only in experiments involving tissue cultures in a liquid medium. Second, if the statistical conditions previously discussed are to be fulfilled, the virus population developing in a lesion must be kept confined strictly to the smallest possible area; because of the presence of the agar overlay, this can be achieved only by the plaque method.

In conclusion, the most satisfactory method of clonation is available only for viruses producing plaques. In the case of viruses that must be assayed in animals, clonation may be very difficult to achieve, particularly if the growth of the virus varies greatly from one animal to another and if the methods for assay are of low efficiency. Unfortunately, these are the conditions prevailing for many tumor-producing viruses.

The Genetic Purity of the Clone

A clone may be impure either because of contamination occurring during clonation or because of the presence of mutants spontaneously arising during the growth of the clone. The first point has already been discussed; to discuss the second point, some examples from our recent work will be briefly reported.

We have encountered in our work with poliomyelitis virus a mutant of type-1 virus called *d* (for delayed), which is greatly attenuated for the nervous system of the monkey and has a markedly reduced efficiency of plaque production when the agar overlay is acid, with a *pH* of 6.8 or lower. If plaques are counted on the third or fourth day, an amount of this virus capable of producing 5×10^5 plaques under an alkaline overlay will not have produced any plaques under an acid overlay. Under identical conditions, the wild-type virus called *d*⁺, which is fully pathogenic, will show only a slight reduction in the number of plaques produced (Vogt and Dulbecco, 1956). This system is well suited for a study of the purity of virus clones with respect to the *d* character. In fact, if a *d*⁺ particle is present among 5×10^5 particles of *d* virus, it can be detected by a plating on the acid overlay.

By using this selective technique, it was found that clonal stocks of the *d* virus contain *d*⁺ reversions in a proportion of approximately 1 in 10^4 .

By studying this and other strains of similar properties kindly supplied by A. B. Sabin, it was found that the virus grows slowly under the acid overlay, too slowly to produce plaques within the standard time of four days. In this process of growth, some mutants in the direction of the wild type arise. These grow somewhat faster and are capable of producing a plaque, although with great delay. If these first-step mutants are not *d*⁺, they may in turn give rise to second-step mutants that may be very similar to the wild type. Under the acid medium there is at first a progressive enrichment of the first-step mutants and, later, an enrichment of the second-step mutants. If passages are made under acid overlay, the population becomes greatly enriched with reversions toward the *d*⁺ type (Dulbecco and Vogt, 1956).

This example shows the importance of spontaneous mutability. The mutation frequency per particle per duplication may be small; in poliomyelitis virus type 1 we have measured mutation frequencies of 10^{-4} to 10^{-5} for one of the mutational steps from *d* to *d*⁺, and of 10^{-5} to 10^{-6} for a mutational step from heat sensitivity to heat resistance (Dulbecco and Vogt, 1956). In spite of this basic stability of the virus, the mutants can rapidly overgrow the original type if they are selected for during the growth of the virus.

The reported example shows that powerful selective effects arose from what might appear to be a rather insignificant change in the environmental conditions. This finding teaches us that the selective effect of a certain experimental condition is hardly predictable.

Another recent observation stresses this point. Stocks of the *t'* mutant of poliomyelitis virus type 1, mentioned in the first part of this article, were grown serially in monolayer culture by using large inocula. After 5 such serial passages, 80 per cent of the particles of the last stock were temperature-sensitive. This result was unexpected, since it was thought that the temperature-resistant type might have an advantage over the temperature-sensitive type; it could be explained by the single-cell experiments already mentioned. The explanation lies in the phenomenon of exclusion occurring in cells

mixedly infected with t^s and t^r virus in which the t^s virus excludes the t^r virus. The high multiplicity of infection used in the passages probably was the condition that, by permitting extensive exclusion of the t^r virus at every passage, determined the enrichment of the t^s virus.

In conclusion, it appears that in certain favorable cases adequate techniques are now available for the production of pure viral clones. These may be difficult to maintain, however. The experience shows that a relatively pure clone can be maintained only by using conditions that do not select for (and possibly select against) spontaneously arising mutants. It is likely that these conditions vary from one system to another and, as shown by the reported examples, that they can be determined only by an extensive experimental investigation of the genetic and physiological properties of the system used.

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DOUBLE INFECTION WITH VIRUSES*

By George K. Hirst

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During the past fifty years considerable progress has been made in the control of a number of virus diseases common to both man and domestic animals. The essential elements in this progress have been basically simple and have involved such measures as vector control, vaccination with attenuated or killed virus, or the administration of antibiotics. Control has been achieved in the face of an almost complete lack of information concerning the activity of the virus inside the cell. Even the attenuated strains have been obtained largely by empirical methods. The need for detailed knowledge of intracellular virus behavior has not appeared to be particularly pressing in the control of ordinary virus diseases.

In considering the role of viruses in tumor formation, however, it is obvious that we are faced with new situations. The relationship of the virus to the tumor cell is subtle, and the older methods of virus control almost certainly will not work. Fortunately, at the present time animal virology shows signs of moving out of an era characterized by rough qualitative methods in a direction of more precision, utilizing such new and powerful tools as *in vitro* cell systems and plaque formation.

Work with bacterial viruses has indicated clearly that one of the best means of unraveling the details of intracellular virus behavior is to crossbreed strains by the double infection of host cells. If, by similar methods, we can find out what goes on in the animal host cell, we shall surely have made a solid contribution to the solution of the virus-tumor problem. I shall summarize briefly some of the Institute's own work on this subject. Unfortunately, this work is in a primitive state; it was done with old and, on the whole, unsatisfactory methods, a fact that gives an air of uncertainty and complexity to the results. If we know little about most animal viruses, we know even less about tumor viruses; therefore, our main interest lies in what may be expected from this approach in the future.

Our experiments dealt with the infection of animal host cells by two similar but readily distinguishable types of influenza virus and the investigation of the possible mutual effect of the two types on their respective progeny. The fact that we are able even to discuss such a problem regarding animal viruses is directly attributable to the magnificent work of Hershey and Chase,¹ whose bacteriophage experiments have completely transformed our conception of the nature of the virus particle.

Among other things, these investigators showed that bacterial viruses have a number of genes that are linearly arranged as if on a chromosome and that, under the proper conditions of mixed infection, the genes of one strain

* Some of the findings reported in this paper resulted from investigations supported by Research Grants E-377 and E-675 from the National Institute of Allergy and Infectious Diseases, Public Health Service, Bethesda, Md.

will exchange with those of another in a process called recombination. One of the most striking aspects of this work was that it stressed the unity of viruses with other living forms, thus justifying the assumption that animal viruses may behave in a similar way. Several research groups, including our own, endeavored to obtain evidence of recombination with influenza viruses. F. M. Burnet's laboratory in Australia and ours in New York, N. Y., have been the only ones so far to publish more than isolated papers on the subject. The work done in Australia is by far the more extensive and ambitious, and it covers work with a wide variety of strains. The conclusion of the Australian investigators² is that exchange of characters can occur between viruses in an infected host cell, while work from the Institute has furnished the principal evidence for a phenomenon called "phenotypic mixing" and for the occurrence of a complex form that has been provisionally called a heterozygote.³⁻⁵ The details of these published data are unimportant; I shall confine myself to descriptions of examples from our own work, which have the advantage of relative simplicity and clarity.

In all of the mixed-infection experiments described, the general technique has been similar. A tissue such as the chorioallantoic sac, containing millions of susceptible cells, is exposed to large amounts of two kinds of influenza virus. Presumably, most cells are infected by both strains. The unabsorbed virus is washed off and, after a suitable incubation period, the yield, usually the initial yield, of the infected cells is collected.

In order to examine the character of individual progeny, it is necessary to obtain clones that have been started from single particles. This is done by infecting eggs at limiting dilutions with very small amounts of virus, so small, in fact, that three fourths or more of the eggs are not infected. There is reasonable evidence that, under these circumstances, some of the eggs are infected by a single particle. Frequently, in order to obtain clones from particles of rarer type, one serological class of progeny is suppressed by specific antisera. This method of clone isolation is slow, tedious, and expensive, as well as imperfect, and it has proved useful only in a limited way. The difficulty of being able to tell with certainty which clones are pure without further passage at limiting dilutions obscures the significance of many results. This is true especially when the isolated recombinants are not stable on further passage. Another difficulty is that with this method it is not possible to study the progeny of a single mixedly infected cell.

Of the phenomena to be described, phenotypic mixing is the most clear-cut and the easiest to understand. For the basic experiment, cells are infected simultaneously with both influenza A and influenza B. These two viruses are similar in behavior, they do not exhibit recombination, and they are not related antigenically by any serological test. However, as many as 85 per cent of the progeny from such a mixed A-B infection can react with both antisera, indicating that such particles must contain surface antigens representative of both parental types. Genetically, these particles are not complex, and the genotype is the same as that of one or the other parent since, when they are subcultured, they give rise only to particles with normal unmixed coats. More extensive and precise experiments with bacteriophage

have produced similar results and have furnished clues for a rational interpretation of our experiments.⁵

From a number of sources, it is now possible to piece together a reasonable, although still rough, account of part of the intracellular behavior of influenza virus. By means of the fluorescent-antibody technique, Watson and Coons⁶ have shown quite clearly that the first detectable virus antigens in the infected host cell are in (or on) the nucleus. It seems possible that genetic material may be reduplicated there. By electron microscopy, Morgan *et al.*⁷ could find finished particles nowhere in the cell except at the limiting membrane, and some of the evidence indicates that completion (coating) of the particle and extrusion are more or less simultaneous. Our evidence from double infections contributes to this picture by showing that the coating process is rather nonspecific and that coats will accept material, the formation of which has been stimulated by widely differing viruses. This picture is consistent with a system in which the genetic material is produced in the nucleus and the coating material is produced in a specific way in the cytoplasm, but is applied to the nuclear cores nonspecifically.

The foregoing is a good example of analogous phenomena occurring in both bacterial and animal viruses, and is another case in which the detailed knowledge of phage is of very definite assistance in suggesting a likely interpretation of less conclusive animal-virus experiments. It shows how experiments with mixed infection can increase our knowledge of what goes on inside the infected cell, even though, as in this case, the phenomena are not primarily genetic. It is also important to understand phenotypic mixing so that this reaction will no longer confuse the picture found with true recombination.

The next group of experiments illustrates recombination. These experiments were carried out with two strains of influenza A⁸ that had common antigens and strain-specific antigens, represented by the letters *M* and *W*. One of these strains was highly virulent for the mouse intracerebrally (+), while the other was completely avirulent (-). When mixed infection with *M*- and *W*+ was carried out, the results were as follows:

The initial mixed infection was performed at a high multiplicity to insure the infection of a large number of cells by two types of particles. The yield from this egg was diluted about 10⁸ times and was inoculated into more than 1000 eggs, about 20 per cent of which became infected. Roughly, one third of these infected eggs yielded type *W*, and one third type *M* virus only. The remaining eggs yielded virus of both serotypes. The exact proportion of double and single yielders varied from experiment to experiment and is not important. There is evidence that the majority of all infections was initiated by single particles. If this is true, then those eggs that yielded two virus serotypes must have been infected by some more complex agent such as a heterozygote, a diploid, a doublet, or perhaps an aggregate. We believed that the existing evidence made the last possibility unlikely and, in one series of experiments, this complex form was carried in series throughout a number of passages at limiting or near limiting dilutions.

The most important aspect of this experiment was the fact that the cells

initially infected with $M-$ and $W+$ virus did not yield any recombinants, but mainly parent-type virus plus an obscure complex particle that yielded both serotypes. On subculture, this latter form yielded both M and W virus. The former was again found to be almost entirely parent-type ($M-$), while the W virus was half parent-type ($W+$) and half recombinant ($W-$). The two striking features of this recombination experiment were (1) that nearly all of the recombinants arose from heterozygotes, and (2) that there was a marked asymmetry of the yield in which $M+$ was virtually absent.

In the foregoing experiments the yield of $M+$ was no more than 1 per cent. Another type of experiment was found, however, in which the yield of this rare recombinant was much higher.⁹

The $M-$ virus was partially inactivated by ultraviolet light. The predominantly inactivated suspensions were titered in two ways: (1) directly in the allantoic sac of the egg in the usual way, the result in the individual eggs, however, being confirmed in each case by subculture; and (2) in eggs that were simultaneously infected with $W+$ virus. In the latter method the yield of M virus in each egg was measured by suppressing the contaminating (activating) $W+$ virus with antiserum. Under these circumstances, when tested in the presence of $W+$ virus, the titer of irradiated M virus was occasionally as much as three logs higher than the control. This increase in titer was probably due to the effect of active on otherwise inactive virus.

When the reactivated M strains were tested for virulence in the mouse, it was found that a few had become irregularly virulent by this route. Much more commonly, however, we found that the reactivated M strains were latently $M+$. These strains had no direct effect in mice, but in mixed infection they had the ability to convert $W-$ strains back to fully virulent $W+$ strains, and they did this with very high efficiency. It will be remembered from the previous test that virtually no $M+$ appeared; therefore, in these reactivation tests its predominance suggests quite strongly that genetic exchange may be the mode of reactivation.

A number of other tests that I shall not describe here were carried out with these strains. One reason for the emphasis placed on this pair of strains was the sharpness of the characters being tested. There was never any ambiguity in connection with either the antigenic type or the virulence factor. The W strains in high dilution (10^{-6}) either killed mice or had no effect at all. A number of different crosses and back crosses were carried out with these strains, emphasizing the quantitative yield of different progeny. In spite of this emphasis, the experiments were not especially fruitful in suggesting the basic genetic mechanisms; they suggested, rather, the need for a fresh approach to the situation.

Examination of the work that has currently been done with the recombination of influenza virus reveals numerous examples of apparent exchange of characters—some examples are quite clear, but many are poorly defined. Most experiments involved the crossing of two strains that differed from each other in so many respects (many of them unknown) that we have no right to expect a simple, readily defined genetic result. Now that the first blush of excitement in the discovery of recombination has passed, we are

faced with the more difficult problem of experimenting with it in ways that will reveal the mechanisms of virus replication and the phenomena that take place inside the infected cell.

In order to improve our approach and obtain more adequate quantitative data, we are using plaque formation rather than the cumbersome limiting-dilution method as a means of isolating pure clones. We are also trying to work with pairs of strains that differ from each other in only a few loci. Finding the best strains for use in crossbreeding is perhaps the most critical part of this task. Preferably, these strains should be mutant lines of one or more of our regular stocks. We must be able to show that the mutant strains do not back-mutate at an appreciable rate and that there is no unequal advantage in the growth of either strain in the host cell.

While we have been working with such systems within several viruses for some time, we have not yet obtained satisfactory quantitative data of any value. A few recombinations have been carried out on chick fibroblast monolayers, such as the conversion of nonplaque-forming to plaque-forming strains, but no group of strains with the right characteristics for the desirable, precise quantitative studies has yet been found. Once a proper beginning has been made, perhaps progress will be more steady.

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TRANSPLANTATION IMMUNITY AND SUBCELLULAR PARTICLES

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In this paper I shall develop an argument in support of the hypothesis that there is a continuous circulation of nucleic acid or nucleoprotein in the body, and that the lymphocyte is essentially a vector of nucleoprotein in the blood-borne phase of its circulation. The argument is in two parts: (1) reasons for supposing that there is a continuous stream of deoxyribonucleic acid (DNA) nucleoprotein from most of the nucleated cells of the body toward the lymph nodes and spleen, and that this nucleoprotein or its nucleic acid moiety is incorporated into lymphocytes; and (2) the suggestion that at least one function of the lymphocyte is to restore nucleic acid to the peripheral tissues, thus completing the cycle. The pathway of the circulation is envisaged thus: tissue \rightarrow afferent lymphatics \rightarrow nodes \rightarrow efferent lymphatics \rightarrow blood \rightarrow tissue space \rightarrow tissue.

I must emphasize the fact that my entire argument is highly speculative. Considered piecemeal, an alternative construction can be imposed upon each separate piece of evidence and, even if each separate piece of evidence is rightly interpreted, the way in which it is assembled may be false. To the best of my knowledge, my final hypothesis is not inconsistent with anything now known about the properties and activities of lymphocytes or tissue cells, but this cannot be regarded as a powerful incentive for supposing it to be true. My defense is (1) that the hypothesis possesses a certain intrinsic plausibility and provides a fresh and perhaps illuminating concept of what have been very puzzling conundrums in immunology and the study of tissue growth; and (2) that the ideas put forward are verifiable, either by existing methods or by slight modifications of those methods.

Evidence for the Passage of Tissue Nucleoproteins Toward Lymph Nodes

The idea that there may be a continuous stream of nucleoprotein from the tissues toward the lymph nodes arose as a by-product of my colleagues' and my investigations of the homograft reaction (Billingham *et al.*, 1956a, b; Medawar, 1956).

It has been proved by the technique of "adoptive immunization" that the principal seat of an animal's reaction against a local tissue homograft is the regional lymph node and, to a very much lesser extent, the spleen (Mitchison, 1953, 1954; Billingham *et al.*, 1954). The principle of the test is as follows. A skin or tumor homograft from a mouse of strain A is transplanted to a mouse of strain B, the "primary host*." After about ten days (the time will vary with the tempo of the homograft reaction, which will in turn depend mainly on the genetic disparity between strains A and B), cells expressed from the regional lymph nodes of the primary host are injected

* The symbols A and B are used algebraically; A does not stand for Strong's A strain.

intraperitoneally into a normal mouse of strain B. This "secondary host" will now react upon an A-line homograft exactly as if it had been actively immunized; in other words, it rejects homografts as if it had been immunologically forearmed by an earlier grafting of A-line cells. At present there is no reason to doubt that adoptive immunization is due to the survival and incorporation in the secondary host of living, immunologically activated cells from the primary host. The cells concerned are lymph-node cells, not necessarily lymphocytes. If the transfer could be effected by an inoculum consisting exclusively of small lymphocytes, that is, of cells that are not known to divide, then it might be necessary to think in terms of the transfer of an antibody-forming mechanism, rather than of viable whole cells, from the primary to the secondary host (compare Chase, 1953). This possibility is, perhaps, raised anew by the arguments presented in the latter part of this paper; however, since it does not affect the argument, the problem will be left open here.

We may assume, then, that something antigenic issues from a skin homograft and reaches the regional nodes, presumably via the afferent lymphatics. Unless we are prepared to believe in a metastasis of whole cells from the graft to the lymph nodes, we must assume that what passes from the graft to the node is something smaller than a cell. The antigenic message may be in the form of ultramicroscopic particles or of cellular fragments. It may reach the lymph nodes by free convection in the lymphatics or by carriage within phagocytic cells. In any event, the clearance of particles or of fragments of dead cells via the lymphatics must be thought of as an everyday transaction of normal tissue, and not as a phenomenon peculiar to homografts. It is true that a tissue is disturbed by the act of grafting, and that many more cells may die in the first week or two after transplantation than would have died if the tissue had been left alone; but if the sexes or strains of the donor and the recipient are judiciously chosen, a skin homograft may live for from fifty to one hundred and fifty days before its breakdown (Eichwald and Silmsker, 1955; Barnes and Krohn, 1957). It may live long enough, therefore, to settle down and behave like normal skin.

The most impressive evidence that antigens in some form issue constantly from homografts is contained in an experiment on the abolition of tolerance to which my colleagues and I have referred more than once (Billingham *et al.*, 1956a; Medawar, 1956). Suppose that a mouse of strain B has been made fully tolerant of A-line cells by the technique of fetal or neonatal injection, and that the B mouse now carries an A-line skin homograft of fifty to one hundred and fifty days' standing. This homograft can be destroyed by injecting its B-line host intraperitoneally with cells expressed from the lymph nodes of *normal* B-line mice. Only one obvious construction can be put upon the results of such an experiment: the homograft is continually giving forth antigenic matter to which its tolerant host is unable to respond. Only when the host is provided with immunologically competent cells from normal donors belonging to its own strain, can the antigenic stimulus take effect. Presumably at least some of the injected lymph-node cells find their way into lymphoid tissue itself (Farr, 1951; Mitchison, 1956).

We may conclude, then, that the liberation of antigens and their passage in some form toward the regional nodes are properties of normal tissues. By "antigen" we mean, of course, a substance that behaves antigenically in the genetical context provided by the act of homografting. In describing them as antigens, we are simply labeling them by the only property that enables us to identify them at all. To say that normal tissues liberate substances that act as isoantigens when introduced into an animal that lacks them is not to say that they exercise any function in normal life. We are saying only that they exist.

The problem now arises of the chemical nature of the substances that act as isoantigens when introduced into genetically foreign soil. My colleagues and I (Billingham *et al.*, 1956b) have tentatively come to the conclusion that they are DNA nucleoproteins, and our evidence for this theory can be classified under four headings: (1) anatomical, (2) chemical, (3) biochemical, and (4) circumstantial.

It should be made clear that our work has been done entirely with mice, and that all our tests under headings 1, 2, and 3 take the form of injecting extracts from the tissues of adult mice of a strain A into adult mice of a strain B to learn whether or not the injection accelerates the breakdown of skin homografts transplanted a few days later from A to B. In theory, an alternative method would be to discover which tissue fractions from A mice would cause tolerance when injected into fetal or newborn mice of strain B; but tests of this kind are lengthy and elaborate, and are open to various technical objections (connected with the duration of the antigenic stimulus in the inception of tolerance) that need not be enumerated here.

Anatomical evidence. The substances that cause skin transplantation immunity in mice are apparently confined to the nuclei of cells: cytoplasmic fractions, even when prepared by biochemically "good" methods, are totally inactive. Alternatively, if kidney cells are disintegrated in media in which nucleoproteins are insoluble, then the active matter is found only in the heavy "chromatin" fraction, sedimentable in an ordinary centrifuge at low speeds. In either case, the use of efficient small-scale methods for separating the nuclei or nuclear fractions from tissues makes it quite certain that the activity we have attributed to nuclei is not due to the accidental presence of intact whole cells that have escaped the processes of disintegration.

Chemical evidence. The active matter is unstable to a degree that seems to rule out ordinary proteins and polysaccharides; it is destroyed or very severely damaged by lyophilization, by repeated freezing and thawing, or by heating for 20 min. to 48.5° C. With no other evidence than this, one would tend to think in terms of intracellular enzymes, nucleoproteins, or lipoproteins.

The evidence from studies on solubility is rather more indicative. In its native form, the active matter is clearly insoluble in salt solutions of physiological strength (about 0.15 *M*). It is soluble in water, however, and if efficient methods are used to disperse the highly hydrated gel that is formed when cells are extracted in water, then, contrary to what we first believed, part of the antigenically active matter remains in the supernatant fluid

after centrifugation for 30 min. at $20,000$ to $27,000 \times g$. It is, however, almost completely deposited by centrifugation for 4 to 5 hr. at $130,000$ to $170,000 \times g$. When $MgCl_2$ is added to an aqueous solution to a final concentration of 0.005 to $0.01 M$, the active matter, together with a good deal of other agglutinated cellular debris, is precipitated. This active matter precipitated by the addition of $MgCl_2$ is now insoluble in water. It may be partially purified by being dissolved in $0.25-M$ $MgCl_2$ and centrifuged for 30 min. at $20,000$ to $27,000 \times g$; active matter can be recovered from the supernatant fluid by diluting it with 10 to 15 volumes of water. This and other kindred evidence (for example, the fact that the active matter can be leached out of nuclei by extraction in $2-M$ $NaCl$) is perfectly in keeping with the idea that the active principle is DNA nucleoprotein, although we are still faced with a number of unresolved anomalies, such as the reason for the inactivity of the precipitate produced by adding $NaCl$ to $0.15 M$ to an aqueous solution.

Biochemical evidence. To our great surprise, we found that the activity of nuclei prepared in media containing bivalent cations, or of the precipitate formed by adding $MgCl_2$ in low concentrations to aqueous solutions of cells, is not destroyed by digestion with trypsin. We are now trying to find out what protein fraction is removed by tryptic digestion. One would guess that it is the histone because, after tryptic digestion, the active matter is soluble in salt solutions of physiological strength containing calcium and magnesium ions. On the other hand, the active matter in nuclei can be destroyed by incubation with deoxyribonuclease (DNase). It is, however, by no means as vulnerable to DNase as would be expected of a solution whose active ingredient was DNA. At present we are inclined to think that the DNA is coated to a large extent with protein, and is thus partly protected against what would otherwise be the very rapidly destructive action of DNase. It should be added that ribonuclease (RNase) is ineffective.

The behavior of the active matter under treatment with trypsin and nucleases is clearly analogous to the behavior of plant viruses. We cannot regard the evidence from enzymic studies as fully conclusive, however; the destructive action of DNase might, for example, be due to the presence of highly active contaminants of a different kind.

Circumstantial evidence. Living whole blood is antigenically active, but its antigenic power resides in the leukocyte fraction and is not to be found in red cells, plasma, or platelets. The same is true of the power of whole blood to produce tolerance of skin grafts after injection into newborn chicks; here, too, the active principle resides in the leukocytes (Billingham *et al.*, 1956a).

This is only minor evidence, however. Much more revealing is the fact that the antigens that cause transplantation immunity appear to be fully represented in all the nucleated tissues of a single individual or of the members of a single inbred strain. We attach some importance to the argument that led us to this opinion. An overlap between the isoantigenic constitutions of the different tissues of the body has long been recognized. For example, the injection of a rabbit B with leukocytes from a donor A will

cause B, with all the characteristic symptoms of heightened sensitivity, to reject skin grafts from A. However, as I was careful to point out when this particular observation was published (Medawar, 1946), this experiment proves only that some antigens present in skin epithelium are also present in leukocytes.

The phenomenon of immunological tolerance provides one with a much more powerful and searching method for revealing antigenic similarities. If, for example, the injection of a fetal or newborn animal B with leukocytes from A enables it, in later life, to accept skin homografts from A (or from some other member of A's inbred strain), then we can infer that every transplantation antigen present in the skin is also present in the leukocyte. Let us suppose that the antigenic make-up of leukocytes is *abcde* and of skin is *cdefg*; in this case the injection of leukocytes into very young animals could not possibly cause tolerance with respect to the antigens *fg*, for these antigens are *ex hypothesi* absent. A skin graft transplanted later in life should therefore be rejected. In fact, as we have pointed out (Billingham *et al.*, 1956a), it is not.

Two other examples from the rapidly growing literature will serve to suggest that the rule is generally valid. Dizygotic cattle twins that have become red-cell chimeras as a result of the exchange of blood-borne cells in fetal life will accept, not only skin grafts from each other, but also grafts of whole functional kidneys (A. Gammeltoft, A. Neimann-Sørensen, and M. Simonsen, cited by Simonsen, 1955). The injection of newborn A-line mice with cells from the spleens of CBA donors will cause them in later life to accept homografts of adrenal cortical tissue from CBA donors (Medawar and P. S. Russell, unpublished).

In the light of evidence of this kind, it looks very much as if the "transplantation isoantigens" are represented uniformly in all the nucleated tissues of the body; we are dealing with a genetical mark of identification that is stamped upon each of the individual's constituent cells. This is exactly what we should expect if the isoantigens were, indeed, DNA nucleoproteins. Conversely, any evidence suggesting that the different tissues contained transplantation antigens peculiar to themselves would be severely damaging to the belief that they were DNA nucleoproteins.

It is proper to conclude this review of evidence by pointing out that we have, so far, no concrete grounds for suggesting that genetic and antigenic specificity resides in the DNA moiety of the nucleoprotein molecule. The sodium salts of DNA have so far proved inactive; we have not yet studied the magnesium or calcium salts. It is, of course, tempting to believe that, viruslike, the protein moiety serves mainly to secure "infectivity," that is, ingress to cells, and to protect the DNA from the action of endogenous nucleases. This may be true, but it is a belief that goes beyond our present knowledge.

The evidence from these four sources has led us to formulate the hypothesis that the antigens responsible for skin transplantation immunity are DNA nucleoproteins; by putting the two sections of the argument together, we now propose that the emission (in some form) of nuclear nucleoprotein, its

clearance by the lymphatics, and its entrance into the regional nodes are normal activities of tissues.

It takes no great ingenuity to see that this interpretation may be very much in error. In the first place, the substances that we have extracted may not be DNA nucleoproteins at all, but other nuclear substances physically associated in some close way with DNA. Much more research will be needed before our interpretation can be accepted as certain. Second, the DNA nucleoproteins (if such they are) may not be directly antigenic; as Leo Szilard suggested in the discussion of this paper, they may transform host cells into a semblance of the donor's cells, and the transformed host cells may then manufacture real antigens of a chemically more reputable kind. Third, it may be that DNA nucleoproteins, although antigenic, are not the substances that actually issue from cells. For the reasons briefly discussed by Billingham *et al.*, 1956b, the whole problem of the nature of the antigenic stimulus in transplantation immunity is of great complexity. If, for example, it should turn out that entirely nonnuclear matter can cause transplantation immunity when administered with the appropriate adjuvants, then, as Mitchison has pointed out, we should be obliged to reconsider our view that the "real" antigens are substances that emerge from the nuclei of cells. In spite of these very real objections and qualifications, however, the idea that DNA nucleoproteins are the real isoantigens and do, indeed, reach the lymph nodes in some form is the simplest hypothesis that our present evidence will sustain.

Lymphocytes and the Fate of Nucleoproteins

We may now turn to examine the consequences of supposing that tissue nucleoprotein, once it has reached the lymph nodes and other immunologically reactive centers, is incorporated into the substance of lymphocytes. The evidence that this might be so is entirely circumstantial. When the nucleoproteins are introduced into genetically foreign animals of the same species, they act as isoantigens, which means presumably that they must enter into lymphoid cells and perhaps even into the nuclei of lymphoid cells (the intracellular fate of injected antigens has been reviewed by Haurowitz, 1952). The grounds for believing that lymphocytes can incorporate nucleic acids or large molecular fractions of nucleic acids from sources external to themselves is discussed below.

The rest of this paper is an attempt to frame an answer to the question: what happens to lymphocytic nucleoprotein? The answer to be put forward here is that the nucleoprotein or its nucleic acid component is paid back into the ordinary nucleated tissue cells from which some of it originally came; Hamilton's (1956) hypothesis that it is paid back directly into lymph nodes and is made up into fresh lymphocytes is treated here as only one part (although perhaps a very important part) of this transaction.

The literature and evidence pertaining to the behavior and fate of lymphocytes now represents an accumulation so vast as to be beyond the complete mastery of any one man. However, my present understanding of the evidence may be summarized as follows:

(1) Lymphocytes are poured into the blood stream in numbers that are much more than sufficient to replace daily the total blood population. The average lifetime of a lymphocyte in the blood is a matter of hours.

(2) There is no evidence that lymphocytes disintegrate in the blood; they must therefore leave it, even if, in due course, they reappear.

(3) There is no sufficient evidence that more than a small fraction of the population of lymphocytes circulates around the following systemic pathway: lymph node → efferent lymphatics → blood system → extracellular tissue space → afferent lymphatics → lymph nodes.

(4) Lymphocytes are of ubiquitous occurrence in the extracellular tissue spaces and between, and even within, epithelial cells of every kind*.

(5) Although some fraction of the population of tissue lymphocytes may actually be extruded through epithelial surfaces into, for example, the lumen of the gut (Bunting and Huston, 1921)—the fraction that behaves in this way is probably small (Kelsall, 1946; Andreassen, 1952).

(6) There is, accordingly, some wastage of lymphocytes, as lymphocytes, in the tissues. The wastage may be large, but its magnitude is uncertain, because there is no biological method of telling whether the lymphocytes seen in tissue sections are a semidomiciliary or a transient population.

(7) Small lymphocytes do not divide, or do so very rarely; they are not, therefore, a reproductively self-sufficient population.

(8) Lymphocytes are very highly mobile cells.

(9) The lymphoid tissues and the lymphocytes are directly and intimately concerned with immunological reactions of all kinds, that is, not merely with the immunological systems in which free serum antibodies play a part, but with hypersensitivities and with transplantation immunity.

Against this background we may now turn to the consideration of some more recent evidence that bears upon the problem of the behavior and fate of lymphocytes. This evidence will be classified under two headings: (1) experiments with radioisotopic labeling of lymphocytic nucleic acids; and (2) experiments on lymphocytic drainage.

Experiments with radioactive isotopes. Otteson (1954) labeled the lymphocytic DNA of two human subjects (one very elderly) with P^{32} , and traced the rise and fall of the DNA- P^{32} in the blood lymphocytes (separated from granulocytes). Hamilton (1956) labeled the lymphocytic RNA and DNA of four leukemic patients with adenine- C^{14} , and traced the label in the blood lymphocytes for nearly a year.

In both cases the radioactivity of the lymphocytes rose to a maximum in a few days and then declined, at first rapidly, and then, over a period of hundreds of days, at an almost imperceptibly low rate. From these data,

* See the evidence reviewed by Schaffer (1927), Andrew and Andrew (1949), and Andreassen (1952). In the special case of epidermal epithelium, there has undoubtedly been some confusion between lymphocytes and the perikarya of melanocytes, but lymphocytes can quite certainly be identified in hyperplastic epidermal epithelia. The abundance of lymphocytes in bone marrow and elsewhere in the tissues has led to the widespread but unfounded and, in my opinion, extremely implausible belief that lymphocytes actually change into other kinds of cells; beyond the central nervous system, there can be few cells into which lymphocytes have not been alleged to transform themselves.

acting upon the conventional assumption that DNA is conserved inviolate for the lifetime of a cell, it is possible to calculate the longevity of lymphocytes. Accordingly, Otteson (1954) inferred that lymphocytes were of two kinds: a minority with a mean lifetime of about 4 days, and a majority with a mean lifetime in the range 100 to 170 days. Hamilton (1956) showed that similar reasoning, based upon only the earlier part of his lymphocyte-survival curves, would put their average lifetime at about 85 days; the latter part of his curves gave about 300 days for the half lifetime of the nucleic acid label.

Experiments on lymphocytic drainage. Lymphocytes can be withdrawn from the body by removing either blood or efferent (central) lymph. Sjövall (1936) found that when rabbits were bled repeatedly over long periods the lymphocytes in their blood fell to about one half of their original concentration and then remained stationary at that level. From this and other evidence he inferred that the lymphocytes in the blood are not all newly formed, and that they circulate along what may be called the tissue circuit, that is, from blood into tissue and thence, via the afferent lymphatics, back into the lymph nodes, and so back to the blood.

Mann and Higgins (1950), using the ingenious technique of Bollman *et al.* (1948) for cannulating the lymphatic ducts of anesthetized rats, showed that the numerical output of lymphocytes from a thoracic-duct fistula fell, after the first day, to about one third of the original value. The numerical output was independent of the rate of lymph flow and was not significantly affected by starvation or deprivation of water; nor could the number be restored by the reinjection of plasma or lymph. Mann and Higgins incline to the view that, in draining lymph, they were withdrawing a "perhaps numerically small but somehow physiologically important group of normally circulating lymphocytes," namely, those that, being able to divide, act as stem cells for the production of others. Very recently, Gowans (1957) has confirmed the findings of Mann and Higgins, and has added to it the extremely important fact that the number of lymphocytes emerging from a thoracic-duct fistula could be prevented from falling by reintroducing the lymphocytes intravenously. Cell free lymph had no such effect, nor did lymphocytes that had been killed, or that had at least been rendered incapable of amoeboid movement, by a brief irradiation with ultraviolet light. Gowans points out that the simplest, although by no means the only, interpretation of these results is to suppose that lymphocytes are circulating cells.

From these two sources we now have evidence that (1) lymphocytes must not be thought to consist of expendable matter, for if they are withdrawn from blood or lymph, their blood concentration or numerical output eventually falls; somehow lymphocytes are needed for their own continued presence in full strength; and (2) that the DNA and RNA label in lymphocytes is of remarkable tenacity. Indeed, the assumption that DNA lives as long as the cells that house it can mean only that some lymphocytes, perhaps a majority, live for several hundred days.

Two kinds of hypothesis, each with several variants, can accommodate this evidence.

The first is that the estimated lifetime of the lymphocyte is to be taken at its face value, and that lymphocytes are circulating cells. This hypothesis is certainly in agreement with the evidence obtained from experiments with radioactive isotopes (above); why, then, should we look further?

To my mind the great difficulty involved in a hypothesis of recirculation is its failure to take account of the wastage of lymphocytes as such, that is, of the wastage of lymphocytes considered as whole cells. Even if only a small proportion of lymphocytes disintegrates in the tissues or, as Heiberg (1923) and Ehrlich (1946) have suggested, in lymphoid tissue itself, then the hypothesis of recirculation can hardly sustain the belief that a high proportion of lymphocytes enjoy an average lifetime greater than one hundred days. Having regard to the fact that the lymphocytes in the blood are replaced several times a day, it might be unrealistically generous to assume that only one lymphocyte in every one hundred is lost daily by disintegration in the tissues and lymph nodes or by accidental death. Even if that figure is accepted, however, the average lifetime of a circulating lymphocyte will still be only seventy days; to justify an average lifetime of not less than two hundred days, the figure that DNA labeling seems to suggest, the average daily mortality of lymphocytes must not exceed three or four per thousand. Too much weight must not be given to this argument, however, because *ad hoc* hypotheses can be designed to accommodate any evidence—for example, Otteson's (1954) supposition that, after their formation, lymphocytes may be stored for one hundred days in lymph nodes and issued in daily dribbles.

The second hypothesis that will explain the evidence discussed is Hamilton's (1956), namely, that lymphocytic nucleic acids are reused to make new lymphocytes.* With a few reservations, Hamilton's hypothesis, like the hypothesis of recirculation, explains the facts we have been considering, although it is a sorry reflection on the state of our present knowledge that the ancillary evidence that gives it special plausibility—evidence of the large-scale destruction of lymphocytes within nodes—is just that evidence to which the hypothesis of cellular recirculation must turn a blind eye.

One objection may be raised at once. If lymphocytic nucleic acid is reused to make new lymphocytes, then why, in Gowans' experiments (see above), should not the reinfusion of killed lymphocytes prevent the drop in the numbers of lymphocytes that issue from a continuous thoracic-duct fistula? In fact, it does not. To me, however, for reasons that will be explained below, it seems likely that the mobility of lymphocytes is vitally important to their ability to participate in a nucleic acid cycle, and in Gowans' experiments the lymphocytes were treated in a way that eventually made them immobile. Immobility was, indeed, his criterion of cellular death.

A second difficulty is the fact that, if there is, indeed, a cycle of reutilization of lymphocytic nucleic acids, then the cycle cannot be supposed to operate exclusively on the blood circuit, that is, along the closed pathway: blood \rightarrow nodes \rightarrow efferent lymphatics \rightarrow blood. To assume that it did so would be to neglect completely the wastage by disintegration of some of the

* Interpretations of this kind, in much less specific form, have been considered by many authors.

lymphocytes that leave the blood and enter the tissues; if this wastage were as little as 1 per cent per day then the half lifetime of the labeled nucleic acids could not be as long as several hundred days. There seems to be only one way out of this difficulty, namely, to suppose that, even if a high proportion of lymphocytes do disintegrate in the tissues, most of their nucleic acid somehow finds its way back to the regional nodes*.

My own guess, therefore, is that many lymphocytes do in fact break down and disappear in the peripheral tissues, and that their nucleic acids are reused in ordinary tissue cells. From here, in due course, by the mechanisms suggested in the first part of this paper, the nucleic acids may find their way back to the lymph nodes, to be reincorporated there in lymphocytes and to set the cycle going anew. I suggest, in short, that there is a systemic circulation of nucleic acids, and that, during the blood-borne phase of the cycle and during the phase in which the lymphocytes are free in extracellular tissue space, the lymphocyte is essentially a vector of nucleic acid or nucleoprotein.

The idea that lymphocytes supply something necessary or, at all events, helpful to the growth of tissues goes back to Alexis Carrel. Since his day it has recurred constantly in the literature—most recently, perhaps, in the work of Humble *et al.* (1956). By using a technique of tissue culture that is, in some important physicommechanical ways, a great deal more realistic and lifelike than methods that present cells with an abundance of free fluid space and fibrin networks, these authors give evidence of a particularly close physical association between lymphocytes and dividing cells. They suggest that lymphocytes can actually enter cells; while this might conceivably be an optical illusion, there can be no doubt of the immediacy and intimacy of the relationship. The way in which lymphocytes dive between cells in the later stages of division makes intelligible their penetration of epithelia; and it is clear that the great agility of the lymphocyte is very necessary in its performance of the kind of transaction I have proposed here.

General Discussion

The hypothesis that nucleic acid circulates in the body has been arrived at by piecing together, far from securely, two or three subsidiary lines of reasoning that all may be wrong. No attempt has been made to conceal the fallibility of the arguments at any stage.

The idea, however, has the merit of allotting a general biological function to the lymphocyte and of explaining, or explaining away, its behavior and fate. I have made no assumption about the degree to which nucleic acids may be degraded within cells before they are incorporated and reused, although I certainly assume that DNA-nucleoprotein passes from tissue to lymph nodes in an immunologically and genetically distinctive form. There is very little likelihood that lymphocytic DNA is incorporated and used by tissue cells in fragments sufficiently large to be genetically distinctive for, if

* Evidence suggesting that a fairly high proportion of lymphocytic nucleic acid circulates through the tissues, rather than direct from the blood to the nodes, can be read into Fichtelius' work (1953) on the fall and rise (to a "second top") of labeled lymphocytes in the blood.

that were so, one would expect homografts on tolerant mice, or homografts transplanted from members of donor inbred strains to F_1 hybrids, to acquire the genotype of their hosts. It is true that parental grafts transplanted to F_1 hybrids seem to undergo radical changes of "transplantability" that are not of selective origin (Barrett, 1952; Klein and Klein, 1956), but these changes are, as often as not, in the direction of *loss* of specificity, the opposite to what one would expect if the grafted parental cells were making wholesale use of their host's nucleic acids in fragments of genetically working size.

One direct test of the concept that nucleic acid is reused in the manufacture of lymphocytes would be to ascertain whether the injection of undegraded nucleoprotein could partially restore or prevent the fall in the number of lymphocytes issuing from a continuous lymphatic fistula (see the work of Gowans, cited above). Such experiments would be difficult, because, in order to insure their validity, one must be certain that the injected nucleoproteins reached the lymph nodes whose output was being drained away. At present there is perhaps just one indirect indication that something of the kind might happen. It is now established beyond question that the permanent or semipermanent repair of acute systemic radiation injury (one of the most immediate consequences of which is damage to the lymphoid tissues) can be brought about only by recolonization of the injured subject with viable whole cells (Lindsley *et al.*, 1955; Ford *et al.*, 1956; Mitchison, 1956). Nevertheless, evidence that the numbers of circulating lymphocytes are temporarily restored by the injection of undegraded nucleoprotein should be sought. The work of Cole (Cole and Ellis, 1954) on the temporary repair of acute radiation injury by the injection of splenic nucleoprotein fractions is perhaps indicative of such an action, but its interpretation is obscured by the fact that only myelopoietic spleens seem to contain the necessary factor (in theory, the nucleoprotein from adult cells should work), and this raises the awkward doubt that their results may be attributed to the contamination of their nucleoprotein preparations with living cells.

Although it would be prudent to confine this discussion to the nonspecific functions of lymphocytes and of nucleic acids, it is impossible to neglect the evidence of immunology, for immunological transactions are the only specific activities that lymphocytes and lymphoid tissues are with certainty known to perform. If it can be assumed that the manufacture of anti-substances is associated with cytoplasmic or (less probably) nuclear nucleic acids, and that this activity is somehow preserved in the hypothetical transfer of nucleic acids from cell to cell, then much that is puzzling about immunological phenomena could, at least in principle, be explained. Hamilton (1956) has already pointed out that the repeated reuse of lymphocytic nucleic acids provides a chemically intelligible background for "the continued formation of antibody long after the apparently limited contact between tissues and antigen." Perhaps it also explains the otherwise extraordinary feat of memory embodied in the secondary or anamnestic response. It gives an understandable physical meaning to the long-lived general sensitization of the tissues that can occur with immunological reactions of the kind for which serum antibodies do not seem to be the

effectors and, as Hamilton says, it provides at least one explanation of how sensitivities of this kind can be transferred by inoculations of lymphoid cells. The hypothesis is in close agreement with Haxthausen's (1948) ingenious demonstration that, when skin grafts are exchanged between identical twins, one of whom is sensitive to dinitrochlorobenzene, the "sensitive" graft loses its sensitivity while the other graft acquires it. The hypothesis, as I have outlined it above, makes the homograft reaction itself intelligible as the interposition of a wrong kind of nucleic acid in a nucleic acid cycle—particularly when we recall that, according to modern evidence (Weaver *et al.*, 1955), the host's reaction against homografts is mediated by blood-borne cells. I suspect that a detailed cytological study of the manner in which these cells destroy homografts will provide invaluable information about the way lymphocytes behave in normal life. But these are all second-order speculations, that is, remote inferences drawn from a hypothesis that is itself of a highly speculative character; they only serve to show that the hypothesis is neither empty nor frivolous, even if it turns out to be completely wrong.

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THE ABSENCE OF IMMUNOLOGICAL IDENTITY IN NEOPLASTIC CELLS

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Introduction

The immunological theory of the origin of cancer was first presented in 1954 (Green, 1954). It has been greatly elaborated since that time (Green, 1957), but much evidence in its favor still remains to be cited. I do not intend to repeat material already published; a brief summary of the salient facts is presented in TABLES 1, 2, and 3.

TABLE 1
DEVELOPMENT OF THE THEORY OF ANTIGENIC DEFICIENCY
IN THE CANCER CELL (GREEN, 1953, 1954, 1955, 1957)
Transplanted tumors

Inhibitory effect of coal tar

Isolation of effective compounds:

Naphtho-2':1-2-anthracene	} Noncarcinogenic or feebly so Nontoxic
11:12-Benzfluoranthene	
Naphtho-2':1'-3:4-pyrene	
Phenanthro-1':2'-2:3-anthracene	
1:2:5:7-Dibenzfluorene	} Carcinogenic Toxic
1:2:5:6-Dibenzanthracene	
3:4-Benzpyrene	

Effective only when retained locally or given I.V. Not effective when intra-tumorally.

Effective only when isoantigenic difference between tumor and host. No effect on spontaneous tumors.

Carcinogenic and inhibitory effects closely related. Both enhanced by cholesterol and inhibited by lecithin.

Cortisone abolishes effect. Therefore an immune process.

Plasma cell reactions to tumor-inhibitory compounds. Cortisone inhibitory.

Splenic pulp from carcinogen-treated or tumor-bearing animal inhibits tumor growth.

From these facts it was deduced that carcinogen increased homologous immunity by inducing an isoantigenic change. If so, tissue-specific antigen might be affected.

The previous publications should be read before considering seriously the present supplementary notes. The problems of cancer anemia and tumor enhancement are considered more particularly here, but only in relation to the more important problem of the nature of the neoplastic process in general.

Since there is some confusion regarding the development of the theory, perhaps one point should be emphasized. It was the nontoxic nature of certain polycyclic hydrocarbons having a transplanted tumor-inhibiting action that first drew attention to the fact that polycyclic carcinogens, although toxic, had a specific inhibitory action on transplanted tumor growth. With the aid of cortisone, it was soon recognized that this was an immunological process, and that probably carcinogens and some related compounds combined with protein complexes in the cell to render them isoantigenic to

the host. The completely innocuous nature of the related noncarcinogenic compounds that had this effect was the pointer to their specificity.

As a key to the whole problem, we had long regarded the fact that neoplastic tissues, in striking contrast to normal tissues, could be transplanted with relative ease. Cancer cells must lack, in some degree, the isoantigens

TABLE 2
FURTHER EVIDENCE FOR CARCINOGEN-INDUCED ANTIGENIC CHANGE

Protein binding

In vitro (Creech)—with serum albumin specific *noncarcinogenic* antigens formed; determinant carcinogenic groups bound.

In vivo (Miller—Heidelberger)—specific and nonspecific.

Tumor proteins—no binding with inducing carcinogen.

Binding of serum lipoproteins with benzpyrene.

Appearance of homologous hemagglutinins and lipid antibodies after intensive carcinogen treatment.

Carcinogen-treated skin refractory to antimitotic action of cortisone.

Reticuloendothelial system

Similar RE response including hemolymph nodes, to transplanted tumor, chemical carcinogens, and X ray.

Carcinogenesis inhibited by—RE blockade

Nonspecific general immunizations

Superficial general irradiation

Leukemia—prevention by bone marrow and spleen injections, thymectomy, and foster nursing with mothers of low-incidence strains.

Tissue culture

Carcinogenesis impossible.

Spontaneous malignant change possible—antigenic loss due to nutritional deficiency.

Hormonal hyperplasia may act similarly.

Hormone-mimetic action of carcinogens

Possible evidence of linkage to tissue identity proteins.

TABLE 3
FURTHER EVIDENCE OF ANTIGENIC DEFICIENCY IN TUMORS

Capacity for homologous transplantation—isoantigen deficiency.

Capacity for enhancement—further isoantigenic loss.

Enhancing effect of hydrocarbons related to carcinogens—blocking antibodies produced?

Capacity for invasion—tissue antigen deficiency.

Tumor viruses—deletion of virus—these are cytospecific and replace tissue antigen—immune reaction.

Retention of virus—successful competition for antigenic building blocks.

Soluble protein deletion in chemical carcinogenesis.

Loss of liver lipoprotein during systemic carcinogen treatment.

Immunological evidence—liver (complement fixation and labeled antibody) lymphocyte (plate-gel technique).

Antibody production by tumor—animal and human evidence of antibodies against host red cells.

responsible for homologous immunity. Since carcinogens increase isoantigenic immunity, they could act either by stimulating antibody formation or by increasing isoantigen formation. No evidence could be found for the former hypothesis, so the latter was adopted. If cancer cells lacked, in some degree, isoantigens, might they not also lack, perhaps in greater degree, tissue antigens? The entire picture of the cancer cell is one of progression

toward a formless, unspecialized type, devoid of its normal particular functions. Such functions must be controlled by master proteins that confer on the cell its identity. It is these identity protein complexes that we believe are lacking, in very different degrees, in the cancer cell. Some further evidence in favor of this theory is presented here, but it is a small part of the whole.

Ionizing Radiations

We have attempted a unified concept of the action of carcinogenic agents by assuming that the action of all is directed against specific antigenic complexes. Such an explanation of virus and ionizing radiation-induced cancer was discussed (Green, 1954) and much more fully considered later (Green, 1957). Evidence was cited showing that *general* irradiation like chemical carcinogens, could be either tumor-inducing or tumor-inhibiting, and it was suggested that nonlethal irradiation may produce a specified antigenic change. Sulphydryl compounds tend to antagonize radiation damage just as they do chemical carcinogenesis. Bone-marrow injections prevent the development of X-ray-induced leukemia (Kaplan, 1953), and Lorenz *et al.* (1951) have also shown conclusively that intravenous injection of bone marrow protects mice from the lethal effects of general irradiation. Spleen shielding, splenic implantation, and parabiosis with a nonirradiated rat are all effective (Jacobson, 1952).

It is noteworthy that only reticuloendothelial tissues are protective. Speculation about the nature of the responsible humoral factor continues unabated. It seems clear to us, however, that immunological factors are again concerned. The fact that a small *general* dose of X ray greatly enhances the inhibitory effect of a local dose on the transplanted tumor (Hollcroft *et al.*, 1952) is highly suggestive of this. We have attempted an answer by using methods similar to those that first led to the immunological theory of carcinogenesis. We assumed that, if general irradiation induced isoantigenic change in some tissues, then *pretreatment*, like the chemical carcinogen, might cause ultimate regression of a transplanted tumor. The difficulty, of course, was that the treatment itself reduced the immune response and that, probably for that reason, many of our experiments were negative. However, if the interval between irradiation and transplantation was long enough to allow for some recovery from X-ray damage of the immune mechanism, the indications were that tumor immunity was increased (TABLE 4). Just as in treatment with chemical carcinogen, tumors in the X-ray-treated animals grew and then regressed, indicating accentuation of the tumor antigenic stimulus. Again, quite similarly, cortisone treatment abolished the therapeutic effect, indicating that an immune response was involved. These experiments recall the work of Murphy (1926), who found that prophylactic treatment with small doses of X ray, either generally or at the implantation site, increased resistance to the growth of a transplanted tumor. Significantly, a ten-day interval was needed to obtain the response. Murphy thought the result was due to the lymphocytosis that occurred. It seems now, however, that the induction of an isoantigenic change in

TABLE 4
EFFECT OF GENERAL IRRADIATION ON RESISTANCE
TO A TRANSPLANTED RAT SARCOMA (Rd/3)

General Irradiation (100 r) 24 and 11 Days Before Transplant. Cortisone Acetate (20 mg. S.C.) 3, 2, and 1 Day Before and on Day of Transplant

Treatment	Number of rats	Number with lethal tumor
Irradiation.....	9	1
Irradiation + cortisone.....	9	5
Controls.....	9	6

irradiated tissue offers a more likely explanation. There are many indications that structural alterations in proteins follow irradiation. One of the more recent (Rieser and Rutman, 1956) is the indication of a quite specific modification in the peptide configuration of the fibrinogen molecule.

The immunological effects of irradiation are considered briefly here, partly because of the new evidence available and partly because they may not seem to fit quite so neatly into the immunological theory of cancer induction as do the chemical carcinogens and the oncogenic viruses. From the early stages of this concept we have been impressed by the similarity between serum and irradiation sickness, and it seems likely that both this acute and the more chronic effect of cancer induction have an immunological basis.

Cancer Anemia and Tumor Anti-Red-Cell Agents

Investigation has led us to believe that cancer anemia, together with the frequent occurrence of hemolymph nodes in transplanted tumor-bearing animals, might contain a clue to the immunological role of cancer cells. Although the existence of tumor hemolysins has long been apparent, their possible connection with the anemia of cancer does not seem to have been stressed. This is probably because the hemolysin was thought to be a nonspecific factor common to many normal tissues, and also because the existence of the anemia had long been considered to be adequately explained by hemorrhage, infection, and malnutrition. It is now clear that this is not the whole story and that it actually may be only a small part of it.

In tumor-bearing animals (Greenstein, 1954) the degree of anemia is a linear function of tumor size irrespective of other features, and it must be accepted that the anemia is a direct result of the presence of tumor cells. It is thus surprising that the condition is still considered a secondary result of tumor growth, presumably due to nutritional competition, rather than a direct destructive effect of the tumor cells. This attitude has persisted in spite of the fact that in human cancer it has been convincingly shown that the life of the red cell is usually diminished, sometimes by half or more, and that the degree of anemia cannot be correlated with replacement or destruction of hemopoietic tissue or with the other commonly attributed factors. Moreover, as we shall discuss later, in certain types of cancer such as lympho-

sarcoma and chronic lymphatic leukemia, there is often a positive Coombs test, indicating that the anemia is of an autohemolytic type and that red-cell antibody is being produced.

In considering direct approaches to the theory of antigenic loss, we had early considered the possibility that the tumor cell might react against host cells that normally possessed some part of the antigenic complex deficient in the tumor. One approach was to study the tumor hemolysins for possible evidence that they represented one form of tumor antibody capable of reacting with the red cells of the host. Using a large variety of rat and mice transplantable, spontaneous, and induced sarcomata and carcinomata, we quickly established the fact that they all contained hemolysin in amounts that, although variable, were usually higher than those in the spleen and lung, and much higher than those in the kidney and liver, which showed decreasing amounts in that order. The very low content of the liver contrasted with the fairly high activity of an induced hepatoma. This quantitative difference was suggestive of a functional difference, although it could have been one more expression of rapid cell proliferation.

The work on the distribution, nature, and properties of tumor hemolysins is far too detailed for inclusion here, and only a few salient points will be mentioned. Most of the tests were made with a 30 per cent soluble protein extract, phosphate buffered at pH 6.4, and some were made with a lipoprotein fraction derived from this. Essential preparative details are described by Green and Wilson (1956). Various methods were used for assessing hemolytic activity (HA) but, in general, a visual estimation of the degree of hemolysis of 5 per cent unwashed cells at 37° C. was made over varying periods (1 to 18 hr.), using increasing dilutions of extract. Activity varied not only with the same tumor type, but with the source of the red cell, there being large species, strain (mouse), and individual (rat) differences. Therefore, for detecting differences, pooled material from a group (4 to 6 animals) was tested mainly against the individual cells of all the animals used in the experiment. An empirical method of scoring was used, and thereby the average was found. A more precise time-dilution method was not practicable with labile material of uncertain potency which it was necessary to test immediately after preparation. The common occurrence of hemoglobin breakdown made a colorimetric assay impracticable.

Using similar materials at room temperature on a porcelain tile, the agglutination potency was also observed and, when positive, was checked at 37° C. by the tube technique.

The main purpose of this work was to learn whether these tumor activities were immunological in nature. Several findings suggested that they were. Soluble proteins, lipoprotein, and even phospholipid fractions showed relative and, in some cases, absolute species specificity. Great care in interpretation is needed here, for Gross (1948) found complete specificity and Baldwin (1955) found none. Although the conclusion varies with the method employed, by using a variety of methods and as many species as possible it would seem that the balance of the evidence is heavily in favor of the fact that activity is stronger on homologous cells. Moreover, it is

often stronger on autologous cells of tumor-bearing animals. Such differences are more apparent when the tumor material is compared with a general hemolytic substance such as egg yolk lysolecithin. In some cases heating (56° for 30 min.) appeared to increase the specificity of the hemolysins; in the case of agglutinins it reduced or abolished it. Hemolytic activity, in general, is retained after heating, often being somewhat less in tumor extracts. The activity of normal tissue extracts (spleen, for example) nearly always seems to increase, often greatly; and strong hemolytic activity may be found in heated liver extracts when little or none is found in such unheated extracts. Like normal tissues, tumor extracts often show an increase of agglutinating activity (AA) after heating. On the whole, the results indicate that tumor hemolysin is stable to heat in the range where many antibodies show stability, and there is evidence that, where the hemolysin is weakened, the addition of serum complement is restorative.

Another relevant point is that, in the body generally, HA and AA are greatest in antibody-forming tissues such as the spleen and the lymph nodes: tissues that normally dispose of effete or modified (possibly antigenically) red cells. Moreover, when the animal is challenged with any kind of red cell, even its own, activity rises in the spleen. In the case of heterologous cells, the increase in activity is much more pronounced on the foreign cell, and thus appears to be an immune reaction. Is it not likely, then, that the red-cell destructive power of the spleen is the result of antigenic challenge? If so, the frequently increased potency of the tumor must seriously be considered in this light. The transplanted tumor cell may have acquired this property by repeated contact with many varieties of homologous red cells; if so, a wide-ranging, although relatively species-specific, hemolysin might be predicted.

The essential step in the investigation of this possibility was to challenge the tumor with red cells and to observe its response. This has been done in a variety of ways that are summarized in TABLE 5. With either direct or remote injection of red cells, it soon became clear that the tumor contained species-specific hemolysin and agglutinin. Regardless of their origin, these factors accumulated in the tumor to a greater extent than they did in the spleen (TABLE 6). Still more important was the fact that a quantitative increase in HA and AA occurred after the challenge with homologous and even with autologous red cells. In the latter case blood was drawn from the animal and was injected immediately into the tumor or its host. In such experiments timing presents the greatest difficulties. Tumor activity increased with the time between the first red-cell challenge and death, and negative results were obtained with short periods of exposure. In fact, the absence of normal activity can be said to suggest that neutralization of all the tumor anti-red-cell agents by the injected red cells had occurred.

Efforts were made to give at least two challenging doses with a ten-day interval between the first dose and the sacrifice of the animal. Because this was often difficult with intratumoral injection in transplanted tumors, the following alternate procedure was employed. The tumor was given a primary "sensitizing" injection and was transplanted later, at which time

TABLE 5
SUMMARY OF VARIOUS EXPERIMENTS PLANNED TO DETECT ALTERATIONS IN SOLUBLE
PROTEIN ANTI-RED-CELL HEMOLYSIN (HA) AND AGGLUTININ (AA) ACTIVITIES
AFTER CHALLENGE OF THE TUMOR-BEARING ANIMAL WITH RED CELLS

Tumor	Number of experi- ments	Red-cell type	Alteration in HA	Alteration in AA
Transplanted Rd/3 rat sarcoma	1	Human	+++	++
Transplanted Rd/3 rat sarcoma	1	Fowl	+++	+++
Transplanted Rd/3 rat sarcoma	1	Rabbit	0	0
Transplanted Rd/3 rat sarcoma	3	Homologous	++(2) 0(1)	++(2) 0(1)
Transplanted Rd/3 rat sarcoma	2	Homologous*	++(1) 0(1)	++(2)
Transplanted Rd/3 rat sarcoma	6	Autologous	+(5) -(1)†	++(5) 0(1)
Transplanted Rd/3 rat sarcoma	1	Autologous spleen pulp	++	++
Transplanted Walker rat carcinoma	1	Homologous	++	++
Spontaneous mouse breast carcinoma	2	Guinea pig	+++	0
Spontaneous mouse breast carcinoma	1	Fowl	+++	0
Spontaneous mouse breast carcinoma	2	Autologous	+(2)	+(1) 0(1)
Transplanted mouse lymphosarcoma	1	Fowl	0	+
Transplanted mouse lymphosarcoma	1	Homologous	0	0

* Sensitizing challenge to tumors followed by one or more doses to the animal transplanted with sensitized tumor.

† Absent.

further injections of homologous rat cells were given. In this way it was hoped that the difference in activity between the spleen and the tumor would be more pronounced, since the spleen would have received only a primary stimulus. Such a result was apparently achieved for, particularly after primary intraperitoneal injection, a rise in tumor activity occurred, sometimes without any rise in that of the spleen. With primary intratumoral injection, the difference was not so marked; it may have been that a larger amount of free red-cell antigen was carried over to act as a primary stimulus.

Both transplanted and spontaneous tumors responded to injected red cells when the degree of response was assessed by alteration in HA or AA, or by both. Agglutinating activity was first noted in such experiments and

TABLE 6
TYPE EXPERIMENT: SPECIFIC HEMOLYTIC (HA) AND AGGLUTINATING (AA)
ACTIVITY OF SOLUBLE PROTEIN EXTRACTS AFTER CHALLENGING Rd/3
RAT SARCOMA WITH HUMAN RED CELLS

Tissue	Unheated		Heated	Unheated		Heated	Unheated	Heated
	HA	AA	AA	HA	AA	HA	HA	HA
I.T. challenge				I.P. challenge			No tumor—I.P. challenge	
Tumor.....	+++	+++	+++	++	++	0	—	—
Spleen.....	0	+	+++	+	+	++	+	+++
Serum.....	0	0	—	+	0	—	+	—

Symbols: I.T. = intratumoral; I.P. = intraperitoneal.

is possibly of greater significance than hemolytic activity. In unheated extracts such activity is rarely found, and then only weakly, in nonchallenged tumor tissue. It is much greater and entirely specific with foreign-cell challenge, but it is seen also with both homologous and autologous cells. Heating of the extract usually accentuates the AA, but causes specificity to disappear. AA is also then seen with nonchallenged tumor extracts and with normal tissues (for example, liver). The nature of the heat effect is uncertain for firm agglutination does not occur but, rather, a conglutination that is readily separable by mild force. This conglutination may be important, however, because it is more obvious (often along with true agglutination) in tumor than in normal tissue extracts (perhaps other than the spleen).

Agglutinating activity is certainly related to the often-seen coagulant activity of tumor-soluble proteins, particularly in the "challenged tumors," in hemolytic tests. The red cells may become clumped firmly into a cylindrical mass that floats vertically with one end on or near the meniscus. As hemolysis continues, this mass slowly sinks and disintegrates. The effect is more evident in heated extracts that, in addition, often precipitate, bringing about complete clearing of the previously opaque suspension. Such events are more frequent in challenged-tumor extracts, and are much more frequent in tumor extracts generally than in the spleen and normal tissues. This is also the case with the occurrence of hemoglobin breakdown during hemolysis, and we are examining both the nature of the breakdown products and the cause of its sporadic occurrence.

The chemical nature of these anti-red-cell tumor agents has been studied with the aid of J. W. Westrop and R. Wilson. It seemed to me that, if an immune reaction was involved, the nature of the antigen and antibodies concerned might throw light on tumor immunity in general. The work can be summarized only briefly here, but the outstanding facts are that both HA and AA are associated with tumor lipoproteins and phospholipids. The

lipoprotein fraction of a rat sarcoma and a rat carcinoma showed strong HA at a concentration of 0.5 mg./ml. In fact, it appeared to account for more than the total activity of the soluble protein extract, possibly because in its preparation an antihemolysin agent, known to be present in tumor extracts, is left behind. This lipoprotein fraction showed quantitative specificity for rat cells, was unaffected by heating, and induced hemoglobin breakdown. In two experiments, although the amount of lipoprotein obtained from the tumors of blood-challenged rats was significantly less, its HA was greater than that from the controls.

This lipoprotein fraction did not agglutinate, but a tumor phospholipid fraction did so most powerfully. Crude total phospholipid occasionally showed mild AA that was more evident in challenged rats. When fractionated on silicic acid columns, the first eluate showed very high AA, acting even up to dilutions of one in five million. This cephalin fraction, representing about 45 per cent of the total phosphatide and consisting roughly of 30 per cent serine and 70 per cent ethalonamine phosphatides together with almost the whole of the acetalphosphatides, was the only one showing any AA. Fractions II (serine and ethalonamine phosphatides) and IV (sphingomyelin, lecithin and, possibly, lysolecithin) showed strong HA, particularly fraction IV, while III (mainly lecithin) was inactive. The latter fraction inhibited the AA of fraction I, a property shared with egg lecithin. The cephalin fraction is of great interest, for it showed high species specificity in its AA and thus distinguished itself sharply from egg cephalin, which possessed mild AA, but showed no specificity.

Only the cephalin fraction has been studied in challenged rats, but in two experiments, as compared with the unchallenged controls, it showed increased activity. Like the lipoprotein fraction, on standing in solution, it showed marked instability and its activity diminished rapidly. It seems likely that this activity reflects that of corresponding lipoprotein(s) in the soluble protein extracts. It is one of several indicators of the possibility that the agglutinating tumor factor is distinct from the hemolytic factor(s). Fraction IV showed the strongest HA and was the only phosphatide fraction that gave evidence of chromatographic fixation with serum from corresponding tumor-bearing rats. When the material (after freezing overnight) was tested the next day, this property had completely disappeared. Possibly this fraction contains the phosphatide that, in the form of a lipoprotein complex, acts as a tumor isoantigen. It is possible that one or more of the other active fractions (that are all obtained from the soluble protein extract containing no free lipid) forms part of the hypothetical lipoprotein tumor antibody. It seems that here the cephalin fraction, with its very potent and specific AA, may very well be important. This specificity of action in a phosphatide is certainly an indication of even greater specificity in the corresponding lipoprotein.

Human tumors were apparently exceptional for, while all of ten different rat and mouse tumors were active, seven of ten fresh human cancer extracts were either only slightly or not active at all. They inhibited other tissue hemolysins and also spontaneous hemolysis. It was not surprising, there-

fore, to find that the cephalin fraction showed very high AA, which was inhibited by the lecithin fraction. It appeared that a strong inhibiting factor in human cancer extracts might be responsible for masking the effects of anti-red-cell agents in the tumor. The fact that lipoproteins in stomach carcinoma showed high hemolytic activity seemed to substantiate this view.

This brief review provides a glimpse of the accumulating evidence in support of the hypothesis that cancer tissues respond to red-cell antigens; it also gives a hint of the possible nature of the reacting substances. The most plausible explanation is that the tumor "filters off" or fixes red-cell antisustances that have formed elsewhere. There are several points that conflict with this theory, however. A small point is that splenectomy did not affect the results of the challenging, but of course there were still other sites (lymph nodes, for example) that would respond.

A more important contradiction is the fact that homologous red-cell injection in the normal rat caused only a slight rise in the activity of the spleen, but a significant rise in that of a tumor. There was also no alteration in the splenic activity of unchallenged tumor-bearing rats. Moreover, serum hemolysin, although not always present, was found only in tumor-bearing rats, whether challenged or not; it was not found in normal rats challenged with homologous red cells even when there had been a rise in splenic activity. There was, in general, no direct correlation between splenic and tumor activity. In addition, intratumoral injection usually gave a better response than did parenteral injection.

While this evidence indicates that the antibody is produced *in* the tumor, it does not necessarily mean that the antibody is produced *by* the tumor cell. In fact, the latter theory would seem most untenable, since most of the tumors concerned (breast tumors, for example) were not even derived from cells incriminated in antibody production. It also appears unlikely, however, that antibody-forming cells in the tumor stroma would produce, in any significant amount, antibody to autologous red cells. If the tumor lysins and agglutinins are indeed antibodies, then the only source of antigen is to be found in normal cells, to which the tumor cells could respond only if they lacked such an antigen. Of course this idea would demand a radical extension of our present ideas of the range of antibody-producing cells. There is sound evidence of local antibody production in such various tissues as the cornea (Oakley *et al.*, 1949, 1951, 1954, 1955), the uterus, the breast, and the adipose tissue, but there is nothing to suggest that this is not the result of infiltration by mesenchymal antibody-forming cells. Nevertheless, it seems possible that other types of cells may retain a primitive immune mechanism. No doubt this mechanism will be different from the mechanism responsible for the response of the highly specialized plasma cell to foreign protein. It might be concerned, not with the nuclear antigens, but with the cytoplasmic lipoproteins, for these are apt to induce, *inter alia*, hemagglutinins and hemolysins. We have found repeatedly that immunization of rats and rabbits with normal tissue and tumor phospholipids and lipoproteins usually has this effect. If the tumor cell is then, as we think, deficient in antigenic tissue lipoproteins, it could in theory react against

those host tissues that contain this lipoprotein with which it comes in contact. Again, in theory the normal cell, during fetal development, could have acquired tolerance to antigenically dissimilar cells.

Is there any other evidence that red-cell antibodies are being produced in the tumor-bearing animal? The circulating red cell provides some indication. This cell usually shows more—sometimes much more—sensitivity to the tumor hemolysins. We also obtained some evidence that a lipoprotein fraction from these red cells had much greater hemolytic activity than a similar fraction from normal cells. There is also immunological evidence to show that the red cell is coated with antibody globulin. Working in our laboratories, Marjorie Savigear (1955) found that the red cells of approximately half of the Rd 3 sarcoma-bearing rats gave a positive reaction to rabbit antirat globulin serum, and that the globulin antibody could be eluted. She also noted that extracts of this tumor appear to have a rather specific lytic action on splenic plasma cells (and possibly lymphocytes), which swell, vacuolate, and disrupt quite rapidly. Plasma cells are not seen in the growing transplanted tumors, but they appear in large numbers when the tumors begin to regress. This has always been puzzling, for there is ample reason to incriminate the plasma cell as the main, if not the only, cell concerned with the production of tumor-isoantigen antibodies. It may be, therefore, that the tumor is elaborating a cytotoxin to the plasma cell, which is therefore only histologically in evidence when the tumor, overcome by the host defense, is no longer able to produce sufficient cytotoxin to destroy the bulk of the invading plasma cells. It is significant that the chemically induced tumor does not appear to contain this factor, at least not in the same amount. There is no plasma-cell response to it and possibly, therefore, no efficient stimulus to toxin formation. Thus we have the interesting possibility of a two-way immune traffic in the homologous transplanted tumor—the host attacking the tumor and the tumor attacking the host.

Hemolymph changes in the nodes of tumor-bearing animals may also have relevance here. They are common with rapidly growing tumors, and increased phagocytosis of the red cell is presumably an indication of some abnormality of this cell. Such changes are also commonly seen in nontumor-bearing animals that have received general X-ray treatment or chemical carcinogens (Parsons, 1938). In fact, it is possible that its occurrence distinguishes between a carcinogen and a noncarcinogen (Lasnitzki and Woodhouse, 1944). Although these facts have been overlooked, they are a strong argument for the occurrence of an immune reaction (affecting red cells incidentally) both during carcinogenesis and after tumor formation. Our own experience confirms this, and it is significant that both carcinogenic and noncarcinogenic tumor-inhibitory hydrocarbons have this effect, although it is stronger in the former. The change during carcinogenesis may be due to the formation of autoantigens; the change after tumor formation may be due to the autoantibodies produced by the tumor.

There is, however, other important evidence that, regardless of the nature of the tumor, normal cell destruction takes place in the tumor-bearing animal. It has been realized for some time (Taylor and Pollack, 1942;

Greenstein, 1954) that the anemia invariably present in rapidly growing transplanted tumors is not the result of nonspecific causation by infection, hemorrhage, or nutritional competition but, instead, is the result of an active process associated directly with the tumor mass and increasing linearly with the weight of the tumor. Latterly, a similar position has been reached in regard to human cancer. There is increasing, scattered pathological evidence that the anemia, which is seen (often in a severe form) in over half of advanced cases, is not due to the causes postulated for a century or more. Even the well-known hypothesis of hemopoietic tissue destruction, or displacement, cannot be substantiated (Collins and Rose, 1948; Shen and Homburger, 1951). The accumulation of evidence now indicates that, because the life of transfused cells is much shortened, the anemia is hemolytic in type and that, moreover, the destruction is often exponential in type and therefore is not merely an acceleration of the normal mechanism (Brown *et al.*, 1944; Brown, 1950).

Recently it has become evident that this destructive process is particularly evident in leukemias and malignant lymphomas (Brown *et al.*, 1951; Ross *et al.*, 1951). The latter authors showed that, contrary to previous findings, acceleration of red-cell production is also occurring. Anemia is a special feature in such patients and is often the major cause of disability. It seemed significant to us that this should be so in the group of cancers all broadly derived from elements of the reticuloendothelial system, for it is also a feature of Hodgkin's disease, myelomatosis, and the reticulososes in general. All are derived from cells that, at one time or another, have been potential candidates as antibody producers. There is now direct evidence that, at least in patients with lymphoid tumors, there is a true autohemolytic anemia. In many such cases the red cells give a positive direct Coombs' test, and free antibody of a warm or cold type may be found in the serum (Dacie, 1955). The red cells often show autoagglutination, microphagocytosis, and greatly increased fragility. Very significantly, the process can be controlled by cortisone treatment (Rosenthal *et al.*, 1955). However, evidence of an auto-hemolytic anemia is not confined to these special tumors. In carcinomatosis the survival of the patient's own cells or of transfused cells is, in most cases, greatly diminished (Hyman and Harvey, 1955; Chodos *et al.*, 1956). There are no obvious signs of a true hemolytic anemia, but the presence of distorted or constricted cells may point to increased hemolysis. Shen and Homburger (1951) found a positive Coombs test in only 6 of 197 cases of advanced cancer.

The mechanism of the autohemolytic anemia produced by lymphoid tumors is still unknown, but we could explain it as an active production of red-cell autoantibody by tumor cells that have been challenged by the host red cells. It may be more evident in such tumors either because their cells are more active antibody producers or because they are more likely to share a common antigen(s) with the red cell. Obviously both possibilities are plausible. However, even if the anemia of these special groups could be explained thus, cancer anemias in general still remain to be explained. It is unlikely a priori that another distinctive mechanism is at work, particularly

since there is overwhelming evidence to indicate that active red destruction of a peculiar nature is proceeding in experimental cancer and in the majority of human cancer cases, probably in all cases where the tumor volume has reached a critical point.

On this basis we sought in general cancer cases evidence of the presence of autoantibodies of the type often found in the lymphoid tumors. With the assistance of Jane Wakefield and G. Littlewood, we used the standard Coombs test. However, instead of the maximum antiglobulin titer of the Coombs reagent, we used as the criterion the lower dilutions of such strength as to give usually, but not always, a delayed positive reaction with normal red cells. If the maximum titer of the antiglobulin serum was $\frac{1}{80}$, it was tested at $\frac{1}{10}$ and $\frac{1}{20}$; if it was $\frac{1}{200}$, it was tested at $\frac{1}{20}$ and $\frac{1}{40}$. The results were recorded as a strong positive (++) if agglutination was immediate on mixing; as positive (+) if agglutination occurred within 1 min.; and as weakly positive (\pm) if it occurred any time before the negative control (5 to 10 min.).

TABLE 7
RESULTS OF AGGLUTINATION TESTS WITH ANTIHUMAN GLOBULIN SERA
ON RED CELLS FROM HUMAN CANCER SUBJECTS

Group	Total No.	Per cent positive (++ +)
<i>Leeds</i>		
All cancer.....	178	56
Widespread cancer.....	23	80
Cancer (excluding leukemia and reticulosos).....	141	53
Leukemia and reticulosos.....	37	70
Noncancer patients.....	82	16
Normal.....	61*	3
<i>Sheffield</i>		
All cancer.....	136	31

* Repeat tests on 14 individuals.

The broad findings will be examined first (TABLE 7). Cancer cases, excluding the leukemias and reticulosos, showed a highly significant increase of positive results as contrasted with noncancer cases (53 per cent as against 16 per cent $p > 1000$), and an overwhelming increase over normal healthy subjects (53 per cent as against 3 per cent). Cases with widespread cancer showed a significant increase over all cases (80 per cent as against 56 per cent $p > 50$). The increase in the positives in the leukemia and reticulosis growth (which includes lymphatic and myeloid types, Hodgkin's disease, and myelomatosis) was hardly significant. As a whole, this group accordingly gave no greater numbers of positives than did other cancer cases, although the lymphoid-tumor group alone gave possibly higher figures (70 per cent of 14 cases). Nor was there, except in the lymphoid and myeloma groups, any greater preponderance of true positive Coombs

reactions. However, only a few of the lymphoid group showed a true positive Coombs test, whereas all of 5 myeloma cases did. It may prove highly significant that a tumor of the plasma cell, the prime antibody-producing cell, should produce such a potent red-cell antibody.

Our later results point to a graduation of effect; a strong positive test at the lower serum dilutions continued up to serum titers approaching those of the standard Coombs test. In a few cases, particularly of widespread cancer, the test was positive at the normal serum titer. This has been our experience in all types of cancer, and we think that the greater number of Coombs positive tests in the lymphoid group reflects only a more energetic, but qualitatively similar, response. Otherwise, the major factor in the response of the red cell to the antihuman globulin test appears to be the amount of cancer tissue present. This is possibly the cause of the divergence between the Leeds and Sheffield results. In Sheffield the main source of material comprised ambulant cases attending the Radiotherapy Center, many of whom, from the present standpoint, would be considered noncancer cases. It is suggestive that the 24 known advanced cases of the Sheffield group gave 60 per cent positives as compared with 31 per cent as a whole.

We conclude that the red cell of the cancer patient is much more susceptible to antihuman globulin antibody than is the normal cell, and that this susceptibility increases proportionally with the tumor mass. Some positive tests in noncancer cases are not surprising, since autoimmune reactions are not infrequent following extensive tissue injury, and all the positives were associated with trauma (postoperative) or acute infection.

What is the explanation, and has this any bearing on the mechanism of cancer anemia? The animal and human results strongly suggest that it has. The group of tumors (lymphoid, myelomas, and possibly Hodgkin's) that provides the strongest reacting cells is the group whose normal cell prototypes have been suspected of antibody production. Blood from cases of myeloma not only yielded a strongly positive direct Coombs test, but also a positive indirect test; moreover, the antibody could be eluted. Successful elution was also obtained in many cases of all types of cancer showing a modified positive test; consequently, there was little doubt about its antibodylike nature. It was also certain that this antibody was not antigamma-globulin, because the sera retained full potency after adsorption with the pure substance. It was completely adsorbed by a trace of human serum, and might therefore have been an antialpha- or beta-globulin. As such, it would be akin to the autohemolytic antibody found in acquired hemolytic anemia (Kominos and Aksoy, 1954), and it might similarly be of the cold, incomplete type, because red cells adsorb the eluted antibody better at 0° C. than at room temperature. Antibodies of this type have frequently cropped up in immunological work on chemical carcinogenesis (see below), and we have reason to believe that they are particularly associated with lipid and lipoprotein antigens.

All this evidence supports the view that the anemia of cancer is due to an immunological attack on the red cell of the host. If so, then it becomes still more likely that the tumor hemolysins and agglutinins are true antibodies.

As we have seen, they do not seem to be produced by the host and adsorbed by the tumor. Moreover, there is no firm evidence to indicate that spontaneous tumors do elicit an immune response in the host (Green, 1957). Thus, we have one more rather unexpected link in the chain of evidence showing that antigenic loss is a feature of the cancer cell and, if so, that this loss must be its *raison d'être*.

Tumor Enhancement

I have emphasized the possibility (Green, 1957) that studies on the tumor-enhancing factor (EF) may give a clue not only to the nature of transplantation immunity (that is, the nature of isoantigens), but to the nature of carcinogenic autoantigens. In our view, carcinogens induce antigenic change; permanent enhancement of tumor growth must involve a related process. In fact, enhancement of tumor growth by polycyclic carcinogens and related compounds is not uncommon (Green, 1955). Tumor resistance and tumor enhancement are closely related, and the effects of cortisone and related steroids in abolishing chemically induced resistance (Green, 1953) may be compared with their antagonistic action on the tumor-enhancing factor (Kaliss and Molomut, 1952; Green, 1954). From such apparently paradoxical results it became clear to us that enhancement of transplanted tumor growth by mildly damaged tissues and their extracts was a purely immunological process. Moreover, it was in some way the result of heightened (rather than, as would be expected) lowered antibody production. We have fully reviewed this subject (Green, 1957). We have also demonstrated the probable chemical nature of the enhancing factors (Green and Wilson, 1956). Great enhancement was obtained by either pretreatment with the corresponding tumor lipoproteins or phospholipids. The reason for the effectiveness of both water-soluble (Kaliss, 1955) and fat-soluble (Martinez *et al.*, 1955) factors now seems clear. At present we are fractionating tumor phospholipids in order to find the active lipid(s); this should give a clue to the chemical nature of the antigen(s) concerned and should thereby give a clue to the possibly related antigens that are deleted from the cell during neoplasia. Such work obviously is closely related to that already cited on the nature of the tumor anti-red-cell factors that are lipoprotein in nature.

Meanwhile, it can be reported that the enhancement obtained with lipoprotein and lipid has been maintained for more than eight generations without further treatment. Casey (Kaliss, 1955), using tumor supernatant extracts to break down strain resistance in mice, reports a similar finding. While in similar experiments other workers have not reported this result, its definite occurrence does show the possibility of inducing rapid antigenic simplification by immunological attack. We (Green and Wilson, 1956) have presented strong evidence demonstrating that enhancement is due to the production of tissue-lipoprotein antibodies, and we have suggested that these might be of the nature of blocking or incomplete antibodies. This would be in accord with the fact that some relatively mild damage to the tissue (from heat, cold, mechanical disintegration) that could result in mild antigenic

modification, seems to be a necessary condition in successful enhancement. The splenic enlargement, normally induced by the immune reaction to the transplanted tumor, is absent or only mildly present in a rat bearing an enhanced tumor. A large plasma-cell response, a potent indicator of tumor isoantigen, is present, however, although comparatively (allowing for splenic size) it is reduced. Therefore there can be only a partial blocking of tumor isoantigen by this treatment. Billingham *et al.* (1956) suggest that, in the enhancing-factor preparation, mild injury destroys the nuclear antigen and leaves the cytoplasmic antigen intact. Antibodies to the latter inactivate the nuclear antigens emerging from the graft. This is a very plausible idea, and we (Green and Wilson, 1956) have supported it. Nevertheless, if enhancement, as it seems, may result in permanent antigenic change, (presumably due to the loss of isoantigens) it seems unlikely that this could occur without antibody-antigen union in or on the tumor cell. This presumably results in the emergence of a cell strain that has lost the antigen concerned. The permanently enhanced tumor continues to induce little or no splenic enlargement in its host and is not susceptible of further enhancement. It has thus either lost at least some of its previous isoantigenic components or these components are masked. The way in which a lipoprotein antibody induces a quite rapid change in isoantigenic structure is not known at present. Nucleoprotein must be involved, and there must be some close antigenic relationship between the cytoplasmic lipoprotein and the nuclear nucleoprotein.

I have emphasized at length (Green, 1957) the importance of knowledge concerning the isoantigens, partly because this may help to understand the nature of the even more elusive tissue antigens that we think are intimately concerned in carcinogenesis. If these are modified by carcinogens and become antigenic, a process of antigenic simplification similar to that which occurs in enhancement may follow. The enhancing effect occasionally seen with substances closely related chemically to carcinogens (for example, 11:12-benzfluoranthene) and commonly seen with certain substituted carcinogens (for example, dibenzanthracene isocyanate) may have relevance here (Green, 1955). It may be due to isoantigenic modification, differing slightly from that usually induced by a carcinogen. Instead of the production of antibodies that supplement the normal isoantibody, the production of blocking antibodies might then be induced. Occasionally, particularly when natural immunity is very high, carcinogens, instead of inhibiting transplanted tumor growth, will enhance it. Is this because isoimmunity is augmented to such a peak that adaptation and isoantigenic simplification occurs? The true explanation will probably be very different, but the fact that, under different conditions, these compounds can be either inhibitory or enhancing suggests that some isoantigenic alteration is concerned. The fact that tissue lipoproteins have a strong enhancing action suggests, again, that the specific carcinogen protein-binding may be accomplished with lipoproteins. If isoantigenic modification occurs in this way, then there is a distinct possibility that tissue antigens (tissue-identity proteins) are similarly modified and ultimately lost from the cell. Antibodies to lipoprotein

complexes seem particularly apt to induce cellular adaptation. Perhaps this is so because nucleoprotein antibodies are lethal, whereas lipoprotein antibodies are less lethal or, if incomplete, are nonlethal, thus making it possible for the affected cell to survive sufficiently long for the occurrence of adaptation by antigenic loss. It is in this way that observations on tumor enhancement by lipoproteins and by substances closely related to carcinogens give indirect evidence of the nature of carcinogenesis.

Acquired Tolerance

Acquired tolerance is obviously important as a tool in cancer immunology and may perhaps yield some vital facts about tumor and tissue antigens. It obviously bears an important relationship to tumor enhancement although, since it appears before the immune mechanism develops, its mechanisms appear to be quite different. Enhancement is associated with a heightened immune response to certain antigens; tolerance is associated apparently with a complete absence of response. Is it possible, however, that it is due, instead, to an absence of easily detectable antibody? On this point the result of an experiment in my laboratory by Baruah (1956) was interesting.

In this experiment rats were made tolerant to TAB vaccine by injection on the first day of life. Tolerance was not quite complete, however, because the later injection induced a slight response that, compared with the response observed in the controls, was much diminished. The splenic enlargement and plasma-cell response was just as great as in the controls and was similar to that previously found in many straightforward immunization tests with this vaccine. Baruah has shown that the plasma-cell response runs closely parallel with the serum antibody, and thus the present result was curious.

The hypothesis that follows is highly speculative. Let us assume that in later fetal and early neonatal life there is an immature, rather than a nonexistent immune mechanism and that this develops similar to the antigenicity of fetal tissues, which evolves gradually. When a fetal red cell is antigenic to its mother, the mother usually produces incomplete antibody. The Rh antigen, the first such type to be described, has been thought to be of a lipid nature. If the fetal antigenic stimulus is incomplete, might not its antibody response also be incomplete, rather than absent? Presented with the same stimulus in later life, the conditioned cells might respond by producing the same incomplete antibody, which would either fail to bind effectively with antigen or might even fail to bind at all, and therefore would not be revealed by standard methods.

The idea that all tissue antigens have thus acquired tolerance to one another during fetal life is too fascinating a speculation to neglect until it is disproved by facts. Faults in this mechanism could be related to the development of tissue antigenicity in early life and could thus provide an explanation of embryonic and other tumors of early childhood.

Lipoproteins, Phospholipids, and Cancer Antigens

I have reviewed evidence (Green, 1957), that lipids are important components of tumor antigens. Most of the positive immune serological tests

in transplanted tumor-bearing animals have been performed with alcoholic or other fat-solvent extracts of the tumor as the source of antigen. Incidentally, most of the methods giving positive tests in human cancer have used similar sources of antigen (Green, 1957). Rapport and Graf (1955) and Rapport and Johnson (1955) showed that species-specific antigenic lipids, possibly phospholipids, were present in the mitochondria of a mouse lymphosarcoma. In my own work I have found evidence that lipid and lipoprotein tumor fractions are part of the isoantigenic complex of the tumor (and no doubt normal cell). On many occasions I have immunized rabbits with such fractions and have found complement-fixing, lipid-precipitating, and hemagglutinating antibodies in the serum. In fact, when one uses lipoprotein, such antibodies may be as abundant as in rabbits immunized with whole tumor cells. It may be noted here that immunization of the rabbit with rat-lipoprotein produced rat hemagglutinins; when free lipid was used, however, only rabbit antibody was found. The evidence suggests that lipoprotein tumor antigens are in fact responsible for all the *circulating* antibodies in the transplanted tumor-bearing animal.

TABLE 8
INTENSITY OF THE PHOSPHOLIPID-ANTIPHOSPHOLIPID REACTION, USING
THE PHOSPHOLIPID FRACTION FROM Rd/3 SARCOMA AND
SERUM FROM Rd/3 SARCOMA-BEARING RATS

	Phospholipid sample									
	1	2	3	4	5	6	7	8	9	10
Serum from Rd/3 sarcoma bearing rats.....	+++	++	++	++	+++	++	+	++	++	+++
Normal rat serum.....	++	+	+	++	+	+	+	+	++	+

The avidity of the serum antibodies for tumor phospholipid was first demonstrated chromatographically by Hakim and Green (1955), who used Spalding and Metcalfe's method (1954). Wilson and I (Green and Wilson, 1956) have confirmed and extended this finding (FIGURE 1; TABLE 8). Only the spleen- and lymphoid-soluble proteins showed increased phospholipid-binding power in the tumor-bearing animal (FIGURE 2; TABLE 9). The local draining showed a much stronger reaction than did the remote lymph nodes, and the data leave little doubt that this was an immune type of reaction. After enhancement of tumor growth, the lipid-binding antibody was still present in the spleen to at least the same degree as that in normal tumor-bearing animals (TABLE 10), thus giving further evidence that lipoprotein antibodies were involved.

Baruah has devised another way of demonstrating lipoprotein antibody in transplanted tumor-bearing animals. Using the method of Makari (1955), in which the mouse uterus is sensitized with serum from an immunized rabbit, he showed clearly (FIGURE 3) that tumor lipoprotein reacts specifically

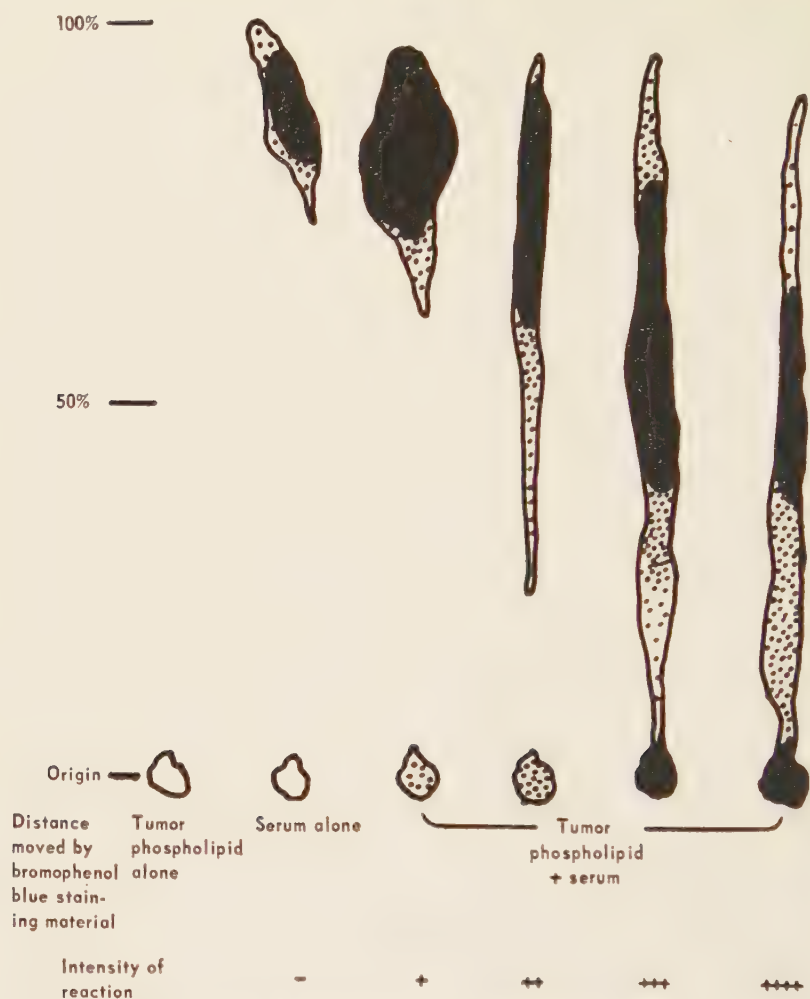


FIGURE 1. Demonstration of the phospholipid antiphospholipid reaction by paper chromatography. Fixation of rat serum by tumor phospholipid.

with tumor lipoprotein antibody. Significantly, no reaction was obtained with serum from rabbits immunized with rat tumor phospholipid. The protein component of the antibody in this case would presumably be rabbit-specific and would not react with rat lipoprotein. It was also shown (FIGURE 4) that this antibody could be transferred passively to the normal mouse uterus by the lymph nodes draining the tumor site. The very specific nature of these effects, separating the tumor antigens sharply from other normal tissue antigens, could easily be interpreted as evidence for a specific tumor antigen. It is more likely to be an indication of tissue specificity and to reflect the important role of lipoproteins in this connection.

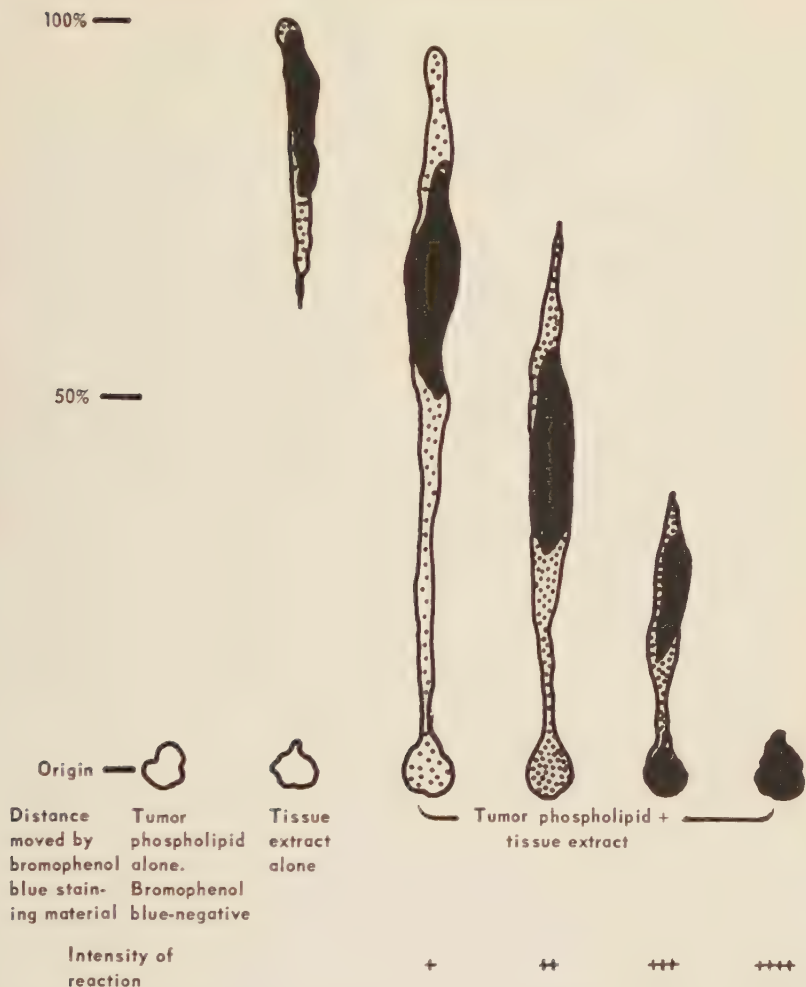


FIGURE 2. Demonstration of the phospholipid-antiphospholipid reaction by paper chromatography. Fixation of the soluble protein fraction of rat tissues by tumor phospholipid.

There is little doubt now that lipoprotein antigens are concerned in tumor isoantigenic immunity. Are they also concerned in the carcinogenic auto-immune process? They well may be. During widespread treatment with chemical carcinogens, homologous hemagglutinins tend to appear in the blood (Green, 1954). This is not the only evidence of a lipoprotein antibody; in a significant number of cases the Kahn test becomes positive, indicating that a lipid antibody with a broad-spectrum character is being formed. It is postulated, then, that chemical carcinogens are capable of modifying cellular lipoprotein so as to render it isoantigenic to its own tissues. There is definitely a tumor-inhibiting factor in the spleen of the carcinogen-treated

animal, and this may prove to be a lipoprotein antibody to this modified antigen. If such isoantigenic modification occurs, a similar alteration in the tissue antigens, with an accompanying autoimmune response, can be envisaged.

Further evidence of possible involvement of phospholipids in tumor immunity has been obtained. Chromatography of the crude lipid (Hakim and Green, 1955) showed that, in general, more "phospholipid" spots were found in transplanted than in spontaneous tumors of the same type; in transplanted tumors, the wider the isoantigenic gap between tumor and host, the more phospholipid spots were found. The most striking difference was in the chromatograms from egg-grown tumors (FIGURE 5). Only

TABLE 10
INTENSITY OF THE PHOSPHOLIPID-ANTI-PHOSPHOLIPID REACTION, USING THE PHOSPHOLIPID FRACTION FROM Rd/3 SARCOMA AND SOLUBLE PROTEIN FRACTIONS FROM THE SPLEEN OF RATS BEARING ENHANCED Rd/3 SARCOMA

Enhancing agent	Phospholipid sample			
	A		B	
	Spleen		Spleen	
	Rd/3 sarcoma-bearing rat	Normal rat	Rd/3 sarcoma-bearing rat	Normal rat
Saline emulsion of frozen Rd/3 sarcoma...	++++	++	+++	+
Lipoprotein fraction from Rd/3 sarcoma...	+++	+	++	+
Phospholipid from Rd/3 sarcoma.....	+++	++	+++	+
Control rats	+++	+	+++	+

lecithin was detected in tumor grown on the developing egg. This seemed to confirm the idea that, as the result of an immune response, new phospholipids were appearing in the tumors. Since then a large number of observations have been made, but we still do not know the precise nature of these spots or the exact conditions determining their appearance. Present evidence suggests that they consist of phosphatide and possibly phosphatide and breakdown products, and that the greater the immune response of the host, the more complex the picture. With the more efficient methods of phospholipid chromatography now available, we hope to obtain evidence as to whether these spots are the result of the union of isoantigen and antibody in the tumor with the release of phosphatide components previously bound in antigenic lipoproteins. Hakim and Green (1955), a few weeks after the injection of a large dose of a carcinogenic hydrocarbon, thought they had detected chromatographically a new phospholipid in the spleen of the rat.

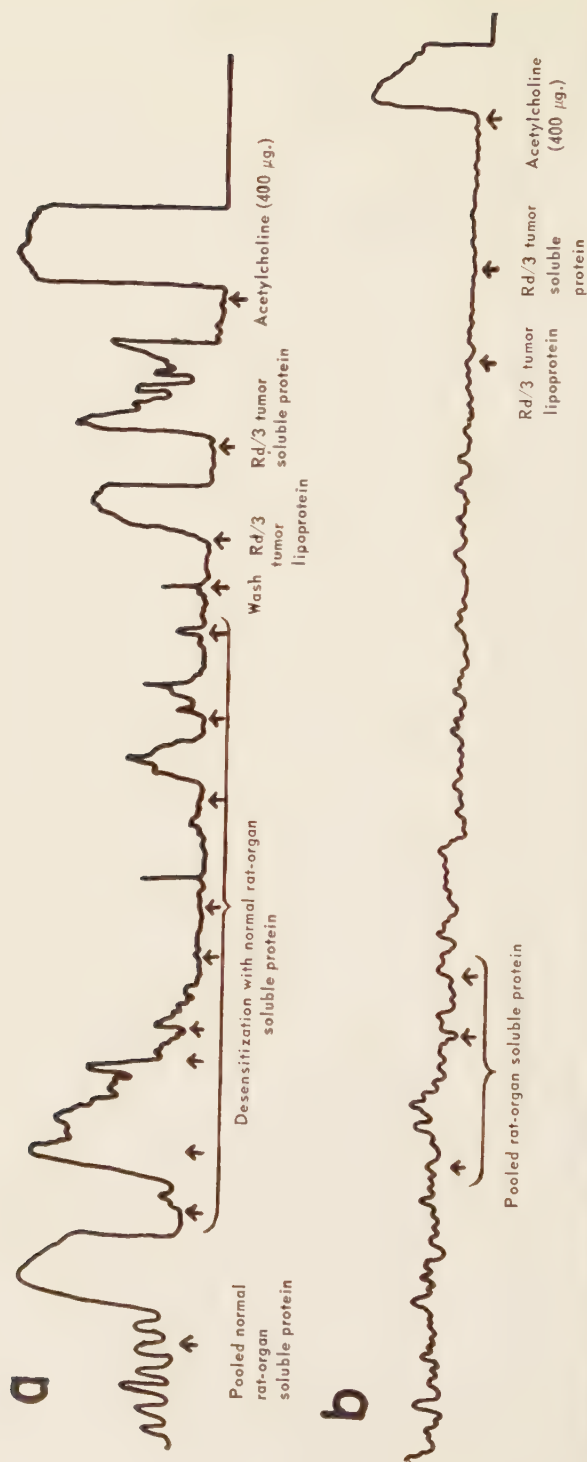


FIGURE 3. (a) Normal Strong A mouse uterus passively sensitized with serum from a rabbit (114) immunized with Rd/3 lipoprotein; (b) normal Strong A mouse uterus, similarly treated, with serum from a normal rabbit.

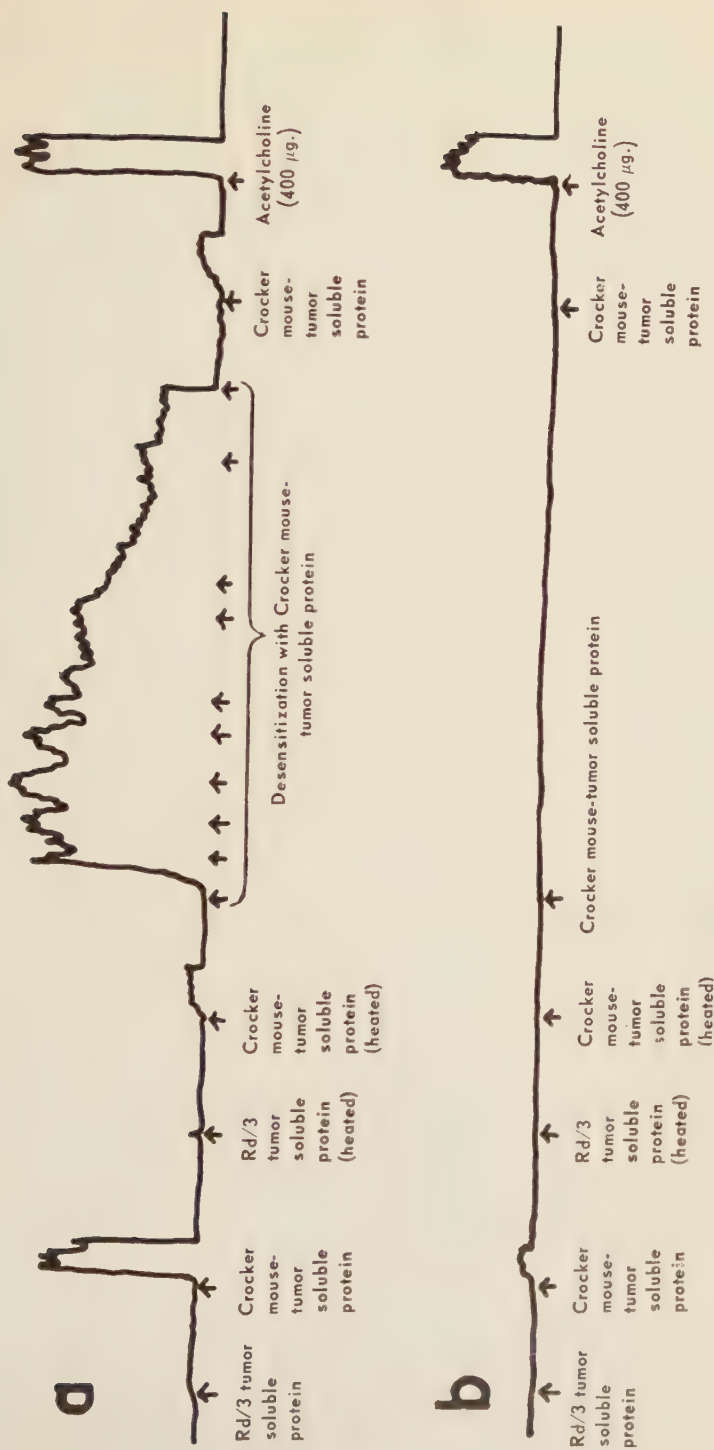


FIGURE 4. (a) Strong A mouse uterus grafted intraperitoneally with draining lymph nodes from Strong A mice bearing growing Crocker tumor; (b) normal Strong A mouse uterus similarly grafted with lymph nodes from normal Strong A mice.

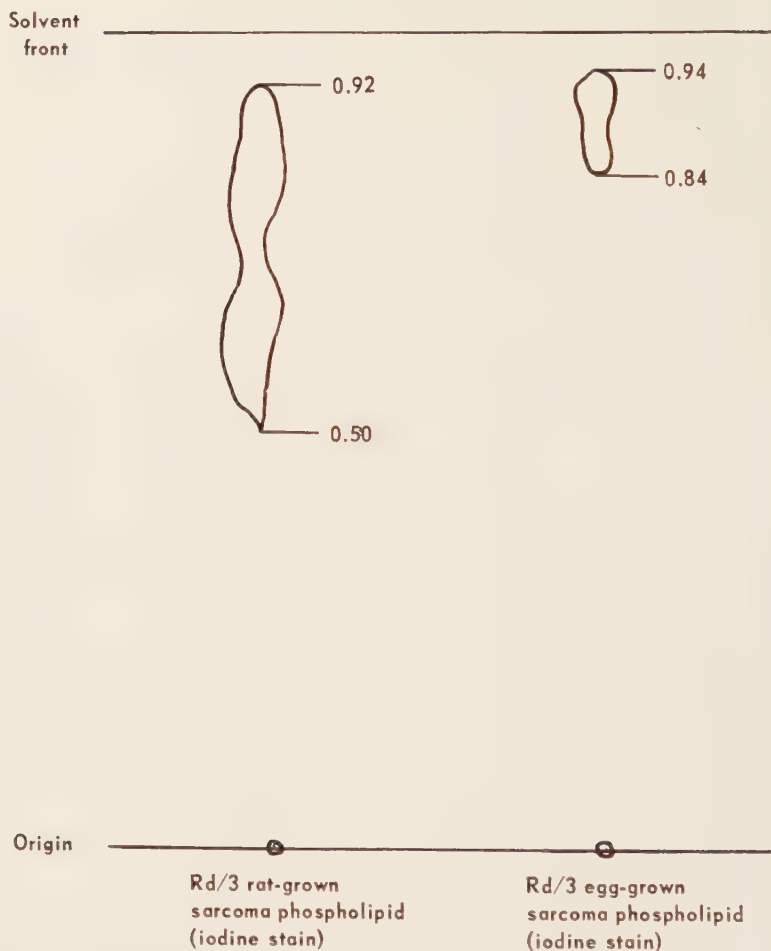


FIGURE 5. Chromatography of the phospholipid fraction from Rd/3 sarcoma. Chromatographic solvent: butanol:ethanol:water = 4:1:5.

Later, other workers in my laboratories were unable to confirm this, although in this elusive field of highly labile phospholipids, with their great variability in activity with minor modifications in treatment, the original finding may still prove valid. If so, it would add substantial support to the concept of the production of an antibody to a carcinogenically modified lipoprotein in the spleen.

The lipoprotein isoantigens may be chemically related to the tissue or organ-specific antigens. Billingham *et al.* (1954) have shown that the immunizing skin-graft isoantigens are nucleoproteins of nuclear origin. Both their and our observations (Green and Wilson, 1956) suggest that the lipoproteins probably are cytoplasmic, structurally related antigens. Despite the fact that they are potent antibody producers, the lipids and lipoproteins

have relatively feeble powers of increasing tumor immunity. In small doses they increase resistance somewhat, possibly by sensitizing the RE system, which then reacts more promptly on receiving a secondary challenge from the transplanted tumor cells. Baruah and I have been unable to find any histological evidence of a tissue reaction to single or repeated injections of lyophilized tumor, tumor phospholipid, or lipoprotein. Unlike the vigorous response seen with living tumor cells or foreign proteins, there is no plasma-cell response, either locally or in the spleen and lymph nodes. It would seem that antibody production is not mediated (at any rate not primarily) through the plasma cell as it is with nucleoprotein. Nevertheless, the two processes appear to be closely related: after primary treatment with lyophilized tumor (lipoprotein antigen), we found a secondary type of plasma-cell reaction to living tumor cells, and Darcy's findings (1955), using frozen tumor, were essentially the same. If the RE system is not the source, or at least not the only source, of lipoprotein antibody, then what other tissues can be considered? Possibly it is a more primitive type of immune response, and all or many types of cells are capable of this production. If so, the ability of tumor cells to produce such antibodies, if the ability exists, would be more easily understood.

However that may be, it seems that these lipid antigens stimulate an immune mechanism that is distinct from the classical one. Indeed, the antibodies induced may not be lethal, and quite likely they may be incomplete. I found that antisera to tumor phospholipids and lipoproteins were not toxic to tumor cells, whereas antisera to living tumor cells were strongly so. If carcinogenesis is due to a specific modification of cellular antigens, then modified isoantigen must be produced to account for the increased homologous tumor immunity induced by carcinogens. In theory, both nuclear and cytoplasmic antigens could be involved. The facts that the spleen and lymph nodes show a plasma-cell reaction to injected carcinogens (Baruah, 1956) and that the spleen, during carcinogenesis, contains a lethal antitumoral factor suggest that nuclear antigen is affected and that this brings about the increased isoantigenic immunity. As we suggested (Green, 1957), the antibody would be lethal to carcinogenically modified cells, some or all of which might die. If not, and if cancer induction continues, it can be assumed that surviving cells would be predominantly those in which the lipoprotein antigens had been solely or mainly affected. By inducing antigenic simplification, the nonlethal lipoprotein antibody to this modified antigen could evoke a new race of cells. The fact that tumor lipoprotein antibody so readily induces adaptation of a related kind in the process of enhancement suggests that this may be a peculiar property of this type of antibody. The fact that the tumor lipoprotein antibody is nonlethal may well account for this property.

A brief reference to the lipid "antibodies" present in the serum of cancer patients is desirable here, because it raises again the question of specific tumor antigens, for which we think there is no firm evidence (Green, 1957). There is no doubt that new lipid-combining substances appear in a majority (often in a substantial majority) of cases. In a lengthy study of such

TABLE 11
BERGER KAHN TEST IN CANCER AND NONCANCER PATIENTS

Type of patient	Number tested	Number positive	Per cent positive
Noncancer.....	441	115	26
Cancer.....	206	91	46

reactions we have found that the Berger Kahn reaction, although not the Wasserman, is positive in a significant percentage of cases (TABLE 11). A small percentage does show complement fixation with other sources of tissue phospholipid. This is, of course, a nonspecific reaction to lipids, and the literature suggests (Green, 1957) that, because tumor lipid is neither essential nor even gives an improved result, this is the case in all such positive serological tests. One possible explanation is that it is an autoimmune reaction to altered lipids released in large amounts from necrotic and damaged tissues. The fact that such tests are often positive in tuberculosis and other widespread infections supports this idea. Positive results are certainly not indicators of a specific tumor antigen. They could, indeed, point, in quite the opposite direction, to the absence of a specific tissue antigen, which would account for the aggressive behavior of the tumor against the host tissues. The results of some of the other cancer-serum "diagnostic" tests may support this latter possibility (Green, 1957). Increased proteolysis with a greater effect on normal rather than cancer tissue is one such result.

Immune Depressant Agents and Carcinogenesis

I have reviewed (Green, 1957) the evidence favoring the view that effective RE blockade inhibits carcinogenesis. The evidence is sound and is supported by our own experiments.

The effects of the immune-depressing agent cortisone may be relevant here. Continued cortisone treatment from the start of skin chemical carcinogenesis sometimes completely suppresses papillomata formation (Ghadially and Green, 1954). This might be because either the immune response or epidermal proliferation or both are suppressed. However, carcinogenic protein-binding (initiation) is not prevented because, before and during the initial stages, cortisone treatment does not prevent the promotion of tumors by croton oil. Epidermal mitosis appears to be suppressed if treatment is started early. However, a curious fact is that, once carcinogenesis is under way, the epidermis becomes much less sensitive to this antimitotic action (Green and Savigear, 1951). If we take the view that all the depressant actions of cortisone on the immune mechanism are due to a specific inhibition of protein (possibly lipoprotein) synthesis, this lack of sensitivity may be due to the emergence of cells that, in some degree, already lacked a target protein. Like all hormones, cortisone presumably owes its specific action to a reaction with proteins that confer identity on the tissue (tissue antigens); if the proteins were modified or lost, the specific hormonal effect

might disappear. This cortisone effect may prove to be an important indication of the specific modification of the identity of proteins in the early stages of carcinogenesis.

Direct Evidence of Antigenic Loss

Our theory relies mainly on a great mass of chemical and biological evidence of immunological activities during carcinogenesis, spontaneous malignancy in tissue culture, and isoantigenic change induced by transplantation and by enhancement, on evidence of an immune response of tumors to autologous cells, and on the general way in which the theory accounts for all the known properties of the malignant cell and the reaction of the host to it. Complete proof demands a demonstration of the absence of specific antigens. This has possibly been demonstrated in at least two instances, in both of which the findings were the unexpected result of a search for cancer antigens. Weiler (1952) showed the absence, in the azo dye-induced hepatoma, of a complement-fixing antibody to normal liver-cell mitochondria. Later (Weiler, 1956) the gradual loss of specific liver antigens was revealed by fluorescein-labeled specific liver-cell antibody. The binding antigen was absent in the frankly neoplastic cell. Seligmann *et al.* (1955), using Ouchterlony's (1948) plate-gel technique with pure suspensions of human leukocytes for rabbit immunization, demonstrated the absence in the malignant lymphoblast of one antigenic component of the normal lymphocyte. No distinctive new antigens were found in leukemic cells of myeloblast or lymphoid origin. Of course, even here there are interpretative difficulties, since the malignant lymphoblast should be compared ideally with the normal lymphoblast. Although the authors do not comment on this finding, taken with the indirect evidence, one positive result is probably a prelude to more.

We have used this technique with animal and human material. One effect we obtained with human material will serve as an example. Rabbits were immunized, with and without adjuvants, with whole-tissue suspensions (for example, stomach, rectal carcinoma, and normal mucosa) and their soluble protein extracts. In the plate-gel tests, deep-frozen soluble proteins from the same source were used as the antigenic source. In FIGURE 6 an effect obtained by G. Littlewood is shown. The normal tissue antiserum

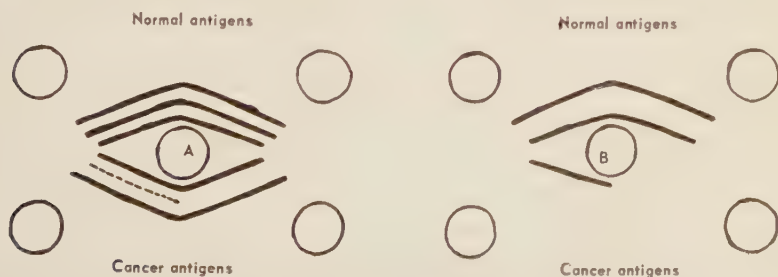


FIGURE 6. Diagrammatic reproduction of precipitin lines obtained with soluble proteins from a human stomach carcinoma and normal mucosa from the same stomach and corresponding rabbit serum antibodies. *A* represents normal antiserum; *B*, cancer antiserum.

showed three components when tested against normal antigens, and only two when tested against cancer antigens (.1 represents the missing component). The cancer antiserum reacted less strongly with .1 in normal tissue and showed little or no reactivity with cancer antigens. This lack of reactivity has been seen in other tests and, while the explanation is not obvious, the whole picture is consonant with soluble antigen loss rather than alteration or gain.

Even in this apparently straightforward test, however, there are many complicating factors. One is the purity of the antigenic source. Obviously, cancer tissue and the corresponding normal tissue cannot be compared easily, because cancer tissue derived from one cell type may be inherently less antigenically complicated than normal tissue derived from several cell types. In fact, an antigen in the cancer cell that is masked by other cells in normal tissue might give a false picture of a specific cancer-cell antigen. Moreover, bacterial and virus contamination is a complicating factor in many human and experimental tumors. In the hope of using pure strains of malignant and normal cells, I am investigating this factor by tissue-culture methods, and also by the use of isolated liver cells (Longmuir and ap Rees, 1956) of normal and neoplastic origin. It can always be objected that comparison should be between the cancer cell and the appropriate normal immature cell; this movement in a circle bedevils cancer research. It should be more fully recognized, however, that if a theory founded on strong experimental evidence squares with most of the facts it should be adopted until it is proved wrong.

Finally, it must be remembered that the chemical evidence of soluble protein deletion during carcinogenesis is indisputable. For this reason I have dealt with it at great length (Green, 1957) as a direct indication of antigenic simplification. The fabric of the immunological theory is doubtless very strong.

Conclusion

It is now clear that the immunological theory of cancer cannot be disregarded. It should be recognized clearly that the undoubted immunological activities of the transplantable tumor apparently have their counterpart in invasion and metastasis by the spontaneous tumor. Capacity for transplantation is related directly to the degree of isoantigenic loss and, for invasion, to the degree of loss of tissue (or organ-specific) antigen. Genetic adaptation of the transplant involves further loss, a loss induced by iso-antibody attack. Of this there is little doubt.

The same principles apply to the spontaneous tumor. Antigenic loss is its *raison d'être*, but the antigens that determine the specific nature of the tissue, and not the individual, are primarily lost. Cells thus affected, when displaced into alien sites, are less easily disposed of by the wandering phagocytic cells because they have lost some of their markers—the tissue antigens or identity proteins. Here again, adaptation (progression) may occur, possibly as a result of a local autoimmune mechanism. We have presented some detailed evidence for this (Green, 1953, 1954, 1955, 1957), but so much

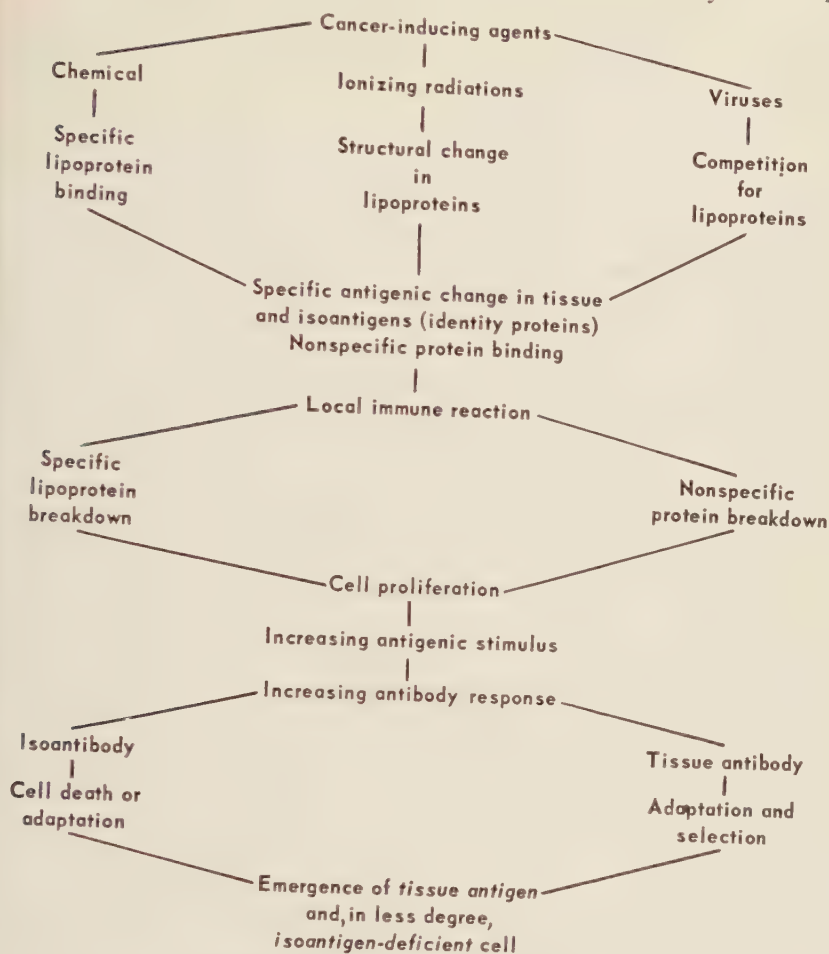


FIGURE 7. General theory of antigenic loss.

Additional evidence has been obtained that the overflow, yet unpublished, accumulates.

Here we have given some concept of the nature of both the isoantigens and, by analogy, the autoantigens concerned; the importance of the phospholipids, particularly in combination as lipoproteins, is demonstrated. Long-term studies have at last revealed the chemical nature of the tumor-enhancing factor, and have shown that cellular isoantigenic loss following immune attack is a reality and that, in this respect, lipoprotein antibodies are peculiarly effective agents. We have previously stressed that many of the important carcinogenic agents are capable of inducing an autoimmune change in susceptible cells. Further evidence has been presented indicating that ionizing radiations can be included in this category. Moreover, the type of immune response elicited suggests that both lipoprotein antigens and nucleo-

protein antigens are affected. The latter induce a lethal antibody and may terminate the process. The former induce a nonlethal antibody and thus cause adaptation by loss of cytoplasmic lipoprotein tissue antigens, and the cancer cell emerges. The outline of a general scheme of this sort is shown in FIGURE 7. Of course this will require many further revisions. It will be seen that, although not an essential feature of the malignant state, allowance has also been made for some degree of isoantigenic loss. Such isoantigenic loss is essential for successful homologous transplantation, and the loss may then be intensified by the immune attack of the host.

The theory explains all the fundamental properties of the cancer cell -- anaplasia, lack of function, invasion, and metastasis. If synthesis of certain specialized protein complexes does not occur, then energy can be directed to the synthesis of less specialized proteins, with a resulting greater growth momentum. It may be this property that enables the induced or spontaneous tumor occasionally to survive long enough in a genetically different host for adaptation to occur.

The theory also explains the neoplastic states induced by viruses and by continued excessive hyperplasia (Green, 1954, 1957). Failure of cells to synthesize tissue antigens due to nutritional deficiency, or to competition (by viruses), or to replacement (by viruses) is postulated. Outgrowth is then possible by a process of selection of the more vigorous simplified cells. The idea can be adequately appreciated only by reference to my other publications. In this connection, the importance of spontaneous malignant change in pure-line cells in tissue culture cannot be overstressed, for here no immune process can be involved. Hyperplasia long continued in an unfavorable environment mimics tissue-culture conditions. In a survey (unpublished), I have shown the importance of both of these conditions in the genesis of many human tumors. Hormonal tumors could arise in exactly the same way, but the intimate relationship of hormones to identity proteins and the hormone-mimetic action of carcinogens, together with their frequency, suggest that they could be induced by a mechanism resembling that of chemical carcinogenesis. To discuss this in full is impossible here, and it suffices to say that, whichever hypothesis of hormone-induced malignancy is adopted, the final stages can be easily explained by antigenic loss. It will also explain the hormonally dependent and independent tumors as based on the degree of loss of identity (target) proteins in the cell. However malignancy is induced, prolonged excessive stimulation of cell growth is essential, either as a promoting agent (that is, gradual increase of the antigenic stimulus) or as a cause, together with a damaged environment, of a deficiency of key metabolites. In neither type of reaction is neoplasia the invariable outcome. In the autoimmune reaction, total death of initiated cells may occur, particularly if isoantigenic change is prominent. This, providing the initiating stimulus is withdrawn, may be a common event. In the deficiency type of reaction, antigenic loss may be partly fortuitous or it may be dependent on some inherited instability of the cell.

The only significantly new proposition (as distinguished from evidence) beyond those previously expressed is that the theory may also account for the aggressive behavior of the cancer cell. The idea foimmunological

attack of an autologous tumor on the host requires a drastic widening of our present concept of immunity mechanisms, which is restricted to certain specialized cell groups. We have shown that the presence of hemolysins and agglutinins in the tumor and red-cell antibodies in the blood may be an indication of this. If so, the cause of the anemia of cancer becomes clear, and its occurrence provides signal proof of the validity of the immunological theory of cancer. Also, intensive study of this anemia should produce vital information about the nature of the antigens involved. The idea could explain both the apparent power of the cancer cell to destroy surrounding normal tissues and the reactive tissue changes (for example, lymphocyte and histiocyte infiltration, fibrosis) that occur. Both would be the result of an immune reaction incited not by the host, but by the tumor. This idea would completely reverse the present approach to tumor immunity. Instead of searching for a specific tumor antigen and a related host antibody, a missing tumor antigen(s), present in corresponding normal cells, and a related tumor antibody would be sought. If the tumor is antigen-deficient, it is the only host-derived tissue in nature known to be capable of such a reaction—all other tissues being nonantigenic to each other through acquired tolerance in fetal development. The biological uniqueness of cancer would thus be explained.

Although we recognize fully the speculative nature of this suggestion, a full review of the immunological literature of cancer (Green, 1957) reveals no firm evidence against it. On the other hand, the clinical course of cancer, at least of certain types, is consonant with it. Tumors derived from the specialized antibody-forming tissues (such as lymphoid, reticular, myeloid and, particularly, the plasma cell) are not only those associated with the severest forms of anemia, but are particularly associated with signs and symptoms that for decades have suggested an infective origin and have led to endless debates as to their inflammatory or neoplastic origin. The recurrent fever and other features of Hodgkin's disease and of other such cases all suggest that a powerful immune reaction is taking place. Since this is not due to infection, is it not possible that it could be one of many examples of the tumor reacting immunologically to its host? Right or wrong, it does not in any way affect the validity of the theory of antigenic loss. It affects only the validity of the hypothesis that all cells are inherently capable of a primitive immune response, perhaps in very varying degrees, when challenged by a *stimulating* antigen. If the theory is wrong, the apparent aggressiveness of the cancer cell can still be explained by the failure of the cell, on having lost identity, to attract the normal disposal mechanism, thus enabling it to survive and proliferate in abnormal sites.

We have reviewed here a few of the more recent findings on the theory of antigenic loss as the *raison d'être* of the cancer cell. The theory is based on experiment and is not entirely theoretical. Without reading all the evidence in its favor (Green, 1954, 1957) a fair appraisalment of it is impossible. Even then it is inevitable that much evidence remains as yet unpublished, for it can be drawn from every branch of cancer studies. The facts it attempts to embrace are legion. The test is to take any fact at random and see if it accords with theory. Having done this ourselves in many diverse fields of

cancer research, of pathology, and of clinical medicine, we are convinced that cancer, from beginning to end, is immunological in nature, and that the theory, first submitted in 1954, is basically true.

Summary

(1) The development of the theory of loss of immunological identity in the cancer cell is summarized.

(2) The process through which this loss is induced is discussed in relation to the carcinogenic effects of ionizing radiations.

(3) Evidence is presented suggesting that cancer anemia, both animal and human, is due to an aggressive immune reaction of the tumor to host cells. If so, here is direct proof of antigenic lack in the tumor cell.

(4) The tumor "antibodies" concerned are lipoproteins in which the phospholipid groups have a strong determinant action.

(5) It is shown that permanent enhancement of transplanted tumor growth by antibodies to tumor lipoprotein points to a similar adaptive mechanism of antigenic simplification during carcinogenesis. Tumor enhancement by hydrocarbons related to carcinogens may be due to the production of closely related antigens and blocking antibodies.

(6) The nature of acquired tolerance is briefly considered and its possible bearing on tumor formation is suggested.

(7) The important role of phospholipid-protein complexes in the antigenic structure of cell cytoplasm is demonstrated in a variety of ways.

(8) Reasons are given for the suggestion that such complexes are modified during carcinogenesis, and that an autoimmune state is induced. Nonlethal autoantibodies cause a specific adaptive antigenic loss and, thus, the state of neoplasia. Formation of lipoprotein antibodies does not appear to be primarily in the reticuloendothelial system and may be a property of many types of cells.

(9) It is suggested that nuclear antigens are also involved, but that severely affected cells are killed by the lethal antibody induced.

(10) Some direct immunological evidence of organ-specific antigenic loss in the cancer cell is presented.

(11) It is concluded that cancer, whether due to defined agents or to a specific nutritional deficiency produced by a faulty environment is, from beginning to end, immunological in nature. Its biological uniqueness is a reflection of the uniqueness of antigenic loss in one cell type of a multicellular organism.

(12) Reviewing the very large body of evidence, including that presented here, it is concluded that the immunological theory, in its original form (Green, 1954), is basically correct. The theory accounts completely for all the special properties of the cancer cell—*anaplasia*, invasion, metastasis, and rapid growth and now, in addition, for aggression.

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AN EVALUATION OF HOST CELL CHANGES ACCOMPANYING VIRAL MULTIPLICATION AS OBSERVED IN THE ELECTRON MICROSCOPE*

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Recent reviews have summarized published electron microscopic studies of viruses in thin sections of infected tissues.¹⁻³ Limitations of space do not allow a detailed account of the rapidly accumulating literature. Suffice it to say that the internal structure of several viruses has been visualized and that some insight into the morphologic aspects of viral differentiation has been achieved. Difficulty has been encountered, however, in ascertaining the effects of these agents on their host cells. Two major problems face the electron microscopist who investigates the response of host cells to viral infection: (1) the distortion introduced during preparation of the specimen and (2) the selection of proper control materials. The former is specifically related to the techniques currently in use, whereas the latter is a basic principle of the experimental method.

Distortion. The fixation of tissue in buffered, osmolar osmium tetroxide with subsequent dehydration in graded dilutions of ethyl alcohol, although producing considerable shrinkage, results in a relatively satisfactory state of preservation.^{4,5} The specimen is then immersed in methacrylate which, after the addition of a catalyst, is polymerized either by heat (40° to 80° C.) or by ultraviolet irradiation. If the shrinkage accompanying polymerization were always uniform, little disruption of cellular components should result. It appears, however, that polymerization may proceed at multiple foci and at differing rates, thus producing variable shrinkage with consequent distortion of the tissue.⁴ The distortion of fine structure is characterized by irregular aggregation of the nuclear matrix, by disruption of the nuclear and mitochondrial membranes, as well as of the lamellae constituting the endoplasmic reticulum, and by displacement of cytoplasmic granules. Factors affecting the degree of damage are under investigation, but at present they remain incompletely understood and thus are difficult to control.

When characteristic "polymerization distortion" is encountered, the specimen is discarded in favor of one that appears to be better preserved. The degree of distortion, however, may vary in different parts of the block or even within a single cell. Unfortunately, therefore, the presence of a well-preserved cell adjacent to a distorted cell may not necessarily mean that the one is healthy and that the other is diseased. Likewise, the presence in the cytoplasm of intact and disrupted mitochondria does not necessarily indicate antecedent cellular damage. Although in healthy cells continuity of individual structures and repetition of their general spatial relationships

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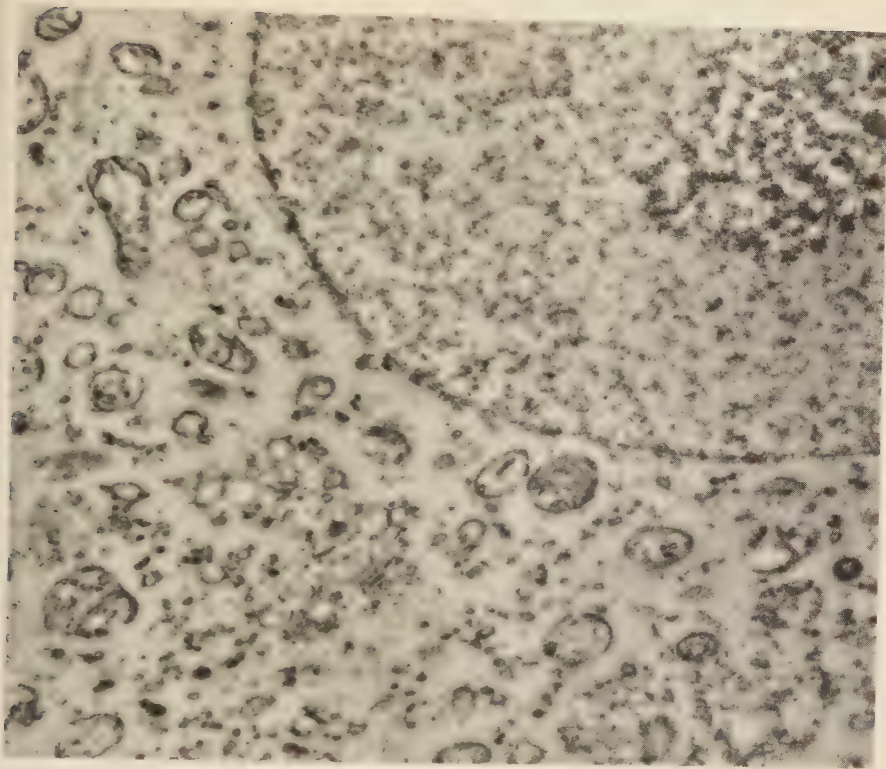


FIGURE 1. HeLa cell from uninfected culture. $\times 15,000$.

may be regarded as criteria of adequate preservation, it is obvious that such criteria cannot always be applied to diseased tissue. In FIGURE 1, for example, aggregation of the nuclear matrix, discontinuity of the nuclear membrane, and disruption of the cytoplasmic components, when viewed together, indicate that "polymerization distortion" has occurred. On the other hand, each of these changes can also occur as a manifestation of disease. Consequently, it is necessary to study in considerable detail the various artifacts that may be introduced during preparation and examination of the specimen* before turning to the investigation of pathological states.

Selection. In many laboratories devoted to electron microscopy attempts have been made to discover the specific effects of a virus on its host cell by comparing infected cells with normal controls. Such an approach can easily result in the accumulation of misleading data. Only by comparing cells infected with different viruses as well as cells undergoing nonspecific† degeneration

* Observations on the distortion produced by the impact of the microtome knife and exposure to the electron beam have been presented elsewhere* and will not be discussed here.

† The term nonspecific is applied herein to those cells in tissue culture that undergo spontaneous degeneration. As the investigation of pathological states is extended the term will undoubtedly acquire a considerably broader application.

ation is it possible to recognize pathological changes that may be significant for the virus under study and that, in certain instances, may be pathognomonic.

Much of this presentation will be devoted to preliminary observations on the morphology of degeneration. The changes to be described are characteristic of a variety of cells but, to maintain continuity, most of the figures will illustrate HeLa cells. Their fine structure resembles that encountered in many normal and neoplastic cell types previously described³⁻⁷ and will not be reviewed at this time.

Degenerative Changes

"Cloudy swelling" is a term widely employed by pathologists to denote certain early changes observed in the cytoplasm after cellular injury.⁸⁻⁹ The nucleus is generally not affected, although margination of chromatin may be encountered.⁹ In the light microscope the cell is seen to be swollen, the cytoplasm assuming a granular appearance. Vacuolization is often apparent. Clear delineation of mitochondrial changes has been impeded by the fact that, relatively early in the process, the mitochondria fail to stain in characteristic fashion and therefore cannot be differentiated from nonspecific granules. Mitochondria have been observed, however, to become swollen and ovoid, thus contributing to the vacuolated or "foamy" appearance of the cytoplasm.^{10, 11} Occasionally they undergo aggregation and may resemble "inclusion bodies" in tumor cells.¹² Not infrequently, lipid globules become more numerous.¹³ By examination of the cytoplasm alone it is difficult to distinguish cloudy swelling, which is reversible, from cellular necrosis. However, certain nuclear changes are considered to indicate either imminent or actual death of the cell. These changes are well known to pathologists; they consist of karyorrhexis (fragmentation), pyknosis (shrinkage with progressively more intense basophilic staining), and karyolysis (diminished density of the matrix with loss of affinity for basic dyes).

In the electron microscope the cytological changes may be visualized in considerable detail, but it would be premature to attempt a precise correlation with the cellular lesions described by light microscopists. An early lesion, originally described by Porter,¹⁴ appears to be fragmentation and vesiculation of the endoplasmic reticulum. In FIGURE 2, for example, the double membranes of the endoplasmic reticulum are distorted and discontinuous, maintaining parallel arrangement for relatively short distances. FIGURE 3 illustrates, at higher magnification, what is believed to be a more advanced stage in this process. The zone separating the lamellae is of variable width. At many points where the reticulum has been interrupted the double membranes have apparently sealed off a vesicle. The linear arrays of vacuoles testify to their origin from lamellar units.

Vacuolization of the cytoplasm and vacuolization and swelling of the mitochondria* have been briefly described by several investigators.¹⁵⁻²¹ Comparisons of cells at many stages of cloudy swelling and necrosis suggest

* Studies have also been made of mitochondria during post-mortem autolysis.¹⁵⁻¹⁷ Whether the changes observed are identical with those encountered in necrosis remains to be determined.

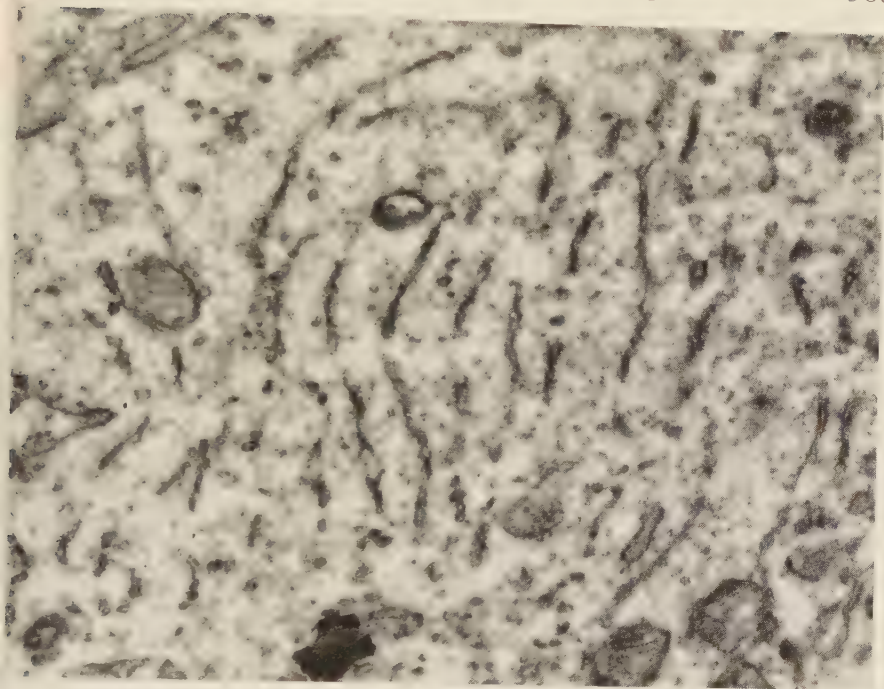


FIGURE 2. HeLa cell from culture infected with adenovirus. $\times 20,000$.

that mitochondria may show a variety of morphologic changes, of which at least three can be clearly distinguished: (1) fragmentation of the cristae into granules; (2) diffuse vacuolization of the mitochondria with disappearance of the cristae; and (3) localized vacuolization with a compression of the cristae. Examples of what are regarded as cases of fragmentation are illustrated by FIGURE 4. In the right third of the electron micrograph, two mitochondria exhibit clearly defined but discontinuous and irregularly disposed cristae. At the lower left corner a swollen, oval mitochondrion still possesses a few membranous structures, while the remainder of its interior is filled by granules of variable size. Scattered through the field are other swollen mitochondria cut at various levels and containing granules believed to represent fragmented cristae. Although these mitochondria seem to possess a single limiting membrane, several vestiges of the second membrane are actually visible in the original micrograph. Apparently both the internal membrane and the cristae undergo fragmentation. FIGURE 5 shows characteristic diffuse vacuolization of mitochondria. In some, no internal structure is visible; in the majority, remnants of cristae are present at the periphery. Mitochondria exhibiting such diffuse vacuolization may show relatively little swelling and may exhibit an irregular folded outer membrane, or they may appear markedly distended. In FIGURE 6 several mitochondria have undergone localized vacuolization. Near the center of the field, two mitochondria possessing a few cristae are probably cut at such a level that their limiting

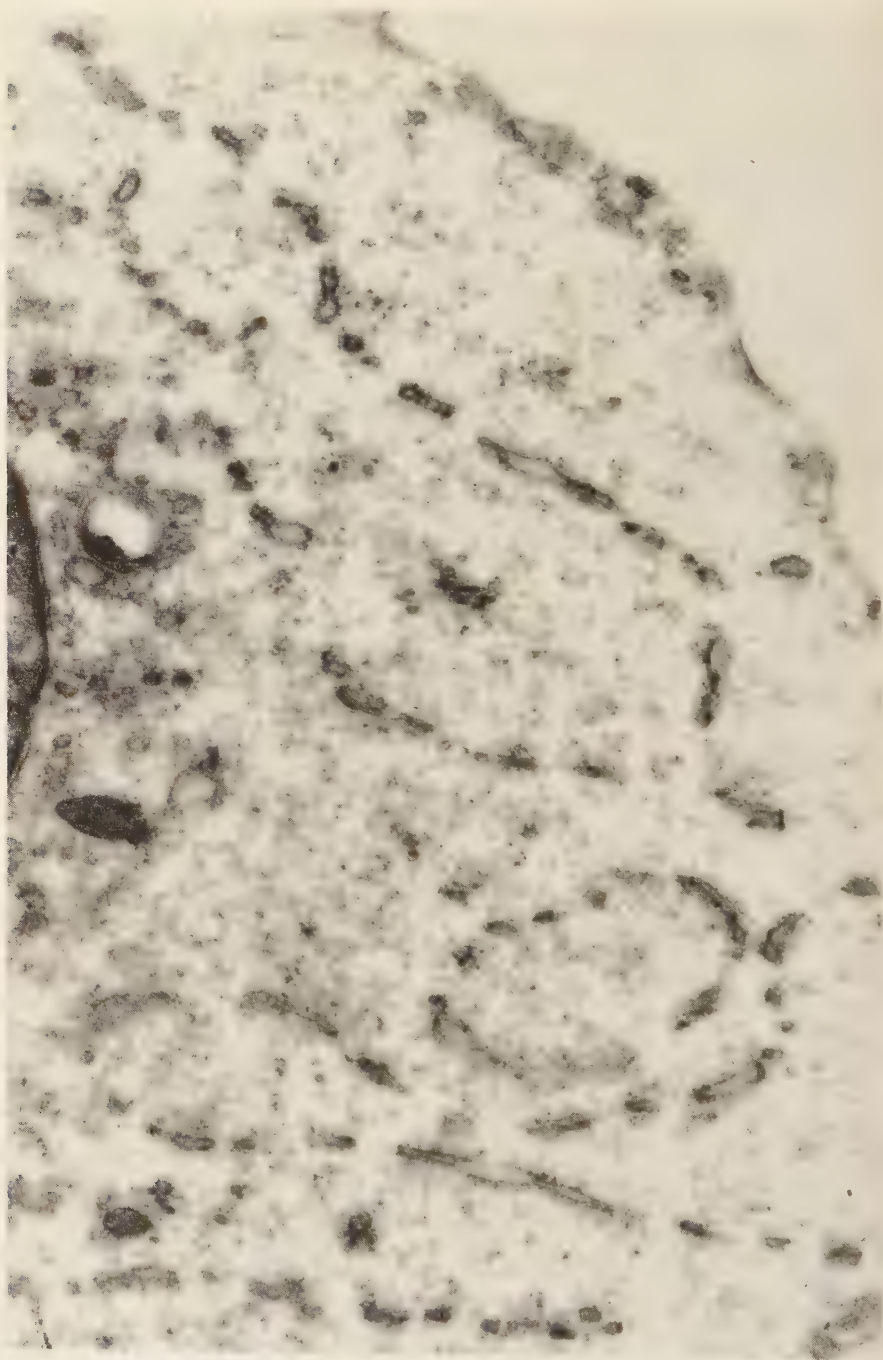


FIGURE 3. HeLa cell from uninfected culture. $\times 26,000$.

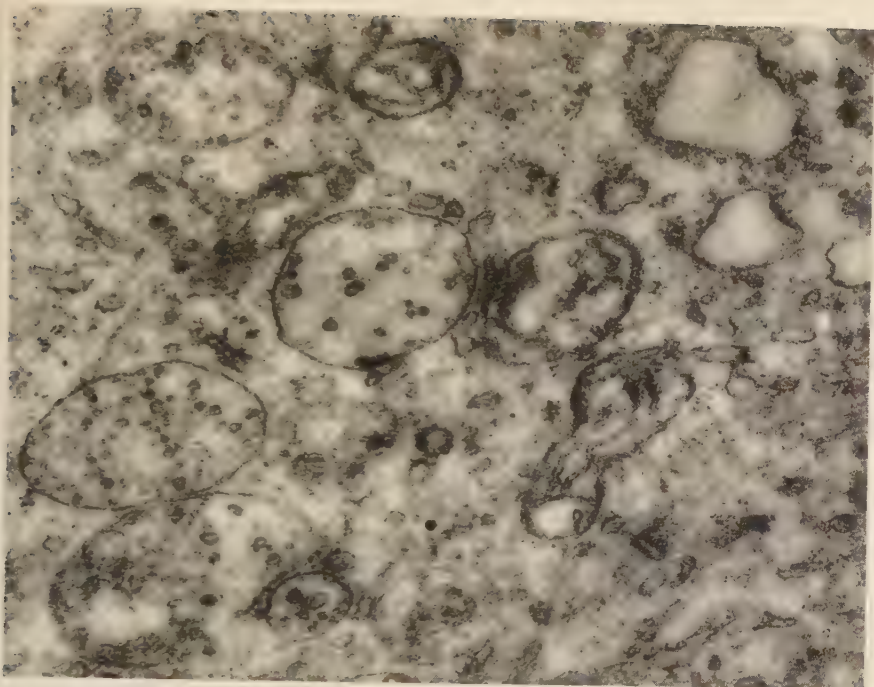


FIGURE 4. HeLa cell from uninfected culture. $\times 40,000$.

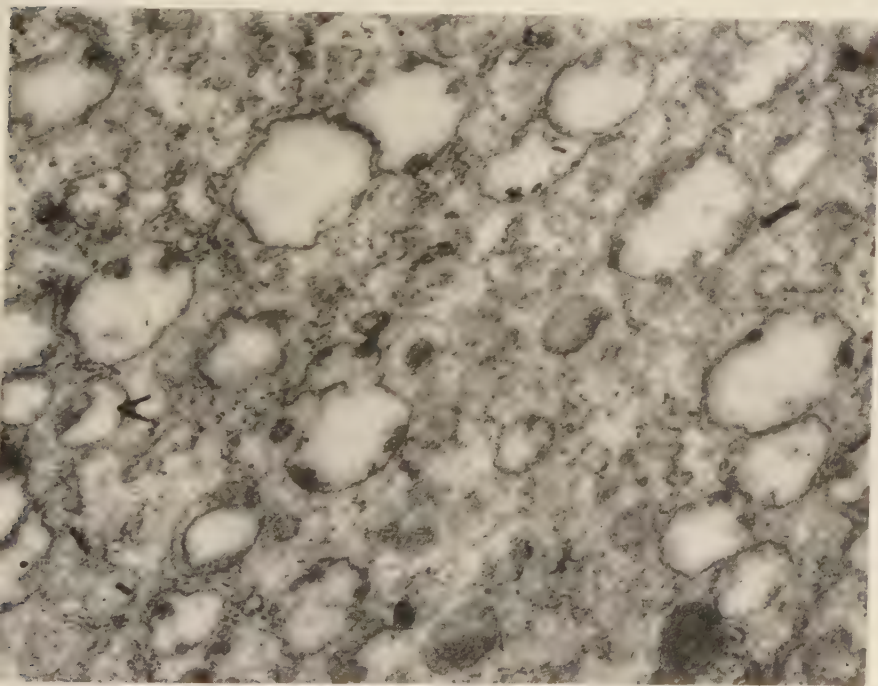


FIGURE 5. HeLa cell from uninfected culture. $\times 23,000$.

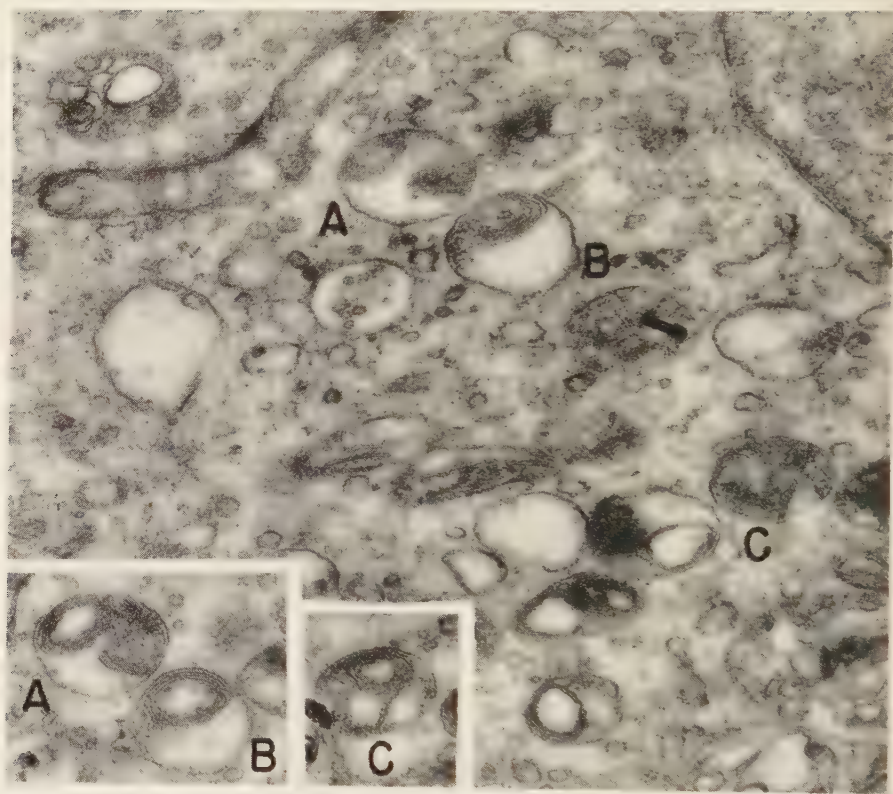


FIGURE 6. HeLa cell from uninfected culture. $\times 35,000$.

membranes are oblique to the plane of section and hence indistinct. Above them a mitochondrion contains granules resembling those previously described. Elsewhere are vacuolated structures believed also to be mitochondria, several of which exhibit internal lamination. When the latter are viewed at the next level in a consecutive serial section (see insets), it is evident that the concentrically arranged membranes enclose vacuoles. Many similar observations support the suggestion that the cristae are compressed into these laminated forms by vacuoles developing within the mitochondria. The vacuolated mitochondrion at the upper left corner might be considered, then, to represent an early stage in this process. It is impossible to tell without extensive serial sectioning whether the large vacuoles scattered through this field are mitochondria that have undergone diffuse vacuolization or that have been cut at such a level that the compressed cristae lie outside the plane of section. The numerous small irregular vesicles are characteristic of those encountered during early stages of cloudy swelling.

As degeneration progresses, some mitochondria disrupt, but others remain intact. Within the latter, condensation of dense granular material appears to take place. FIGURE 7 illustrates numerous mitochondria in differing

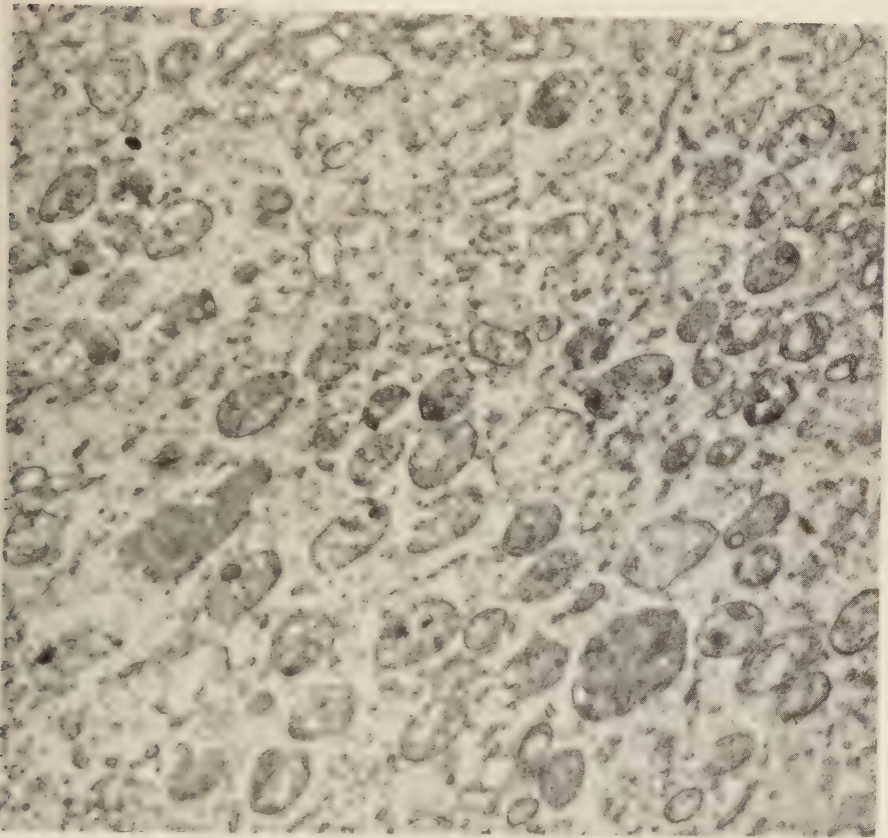


FIGURE 7. Epithelial cell from chicken embryo yolk sac infected with rickettsiae. $\times 15,000$.

stages of disintegration. Some are vacuolated, while others contain the granules presumed to develop from the fragmentation of the cristae. The membranes of several have ruptured, and the granules are in process of release into the cytoplasm. Certain mitochondria contain dense, finely granular material that appears to form a structure relatively resistant to factors causing necrosis of other cytoplasmic components. In FIGURE 8, for example, a number of bodies believed to represent altered mitochondria persist despite lysis of the adjacent cytoplasm. The changes described above are similar in some respects to those observed by Gansler and Rouiller²³ in the cells of animals that had been subjected to starvation. The reasons for the degeneration of mitochondria in a variety of different ways and for the changes that signify necrosis are unclear at present and deserve further study. It has been felt necessary, however, to dwell here at some length on mitochondria, because of the resemblance of certain degenerative forms to aggregates of virus (FIGURES 4 and 7) or to presumed viral inclusion bodies (FIGURE 8).

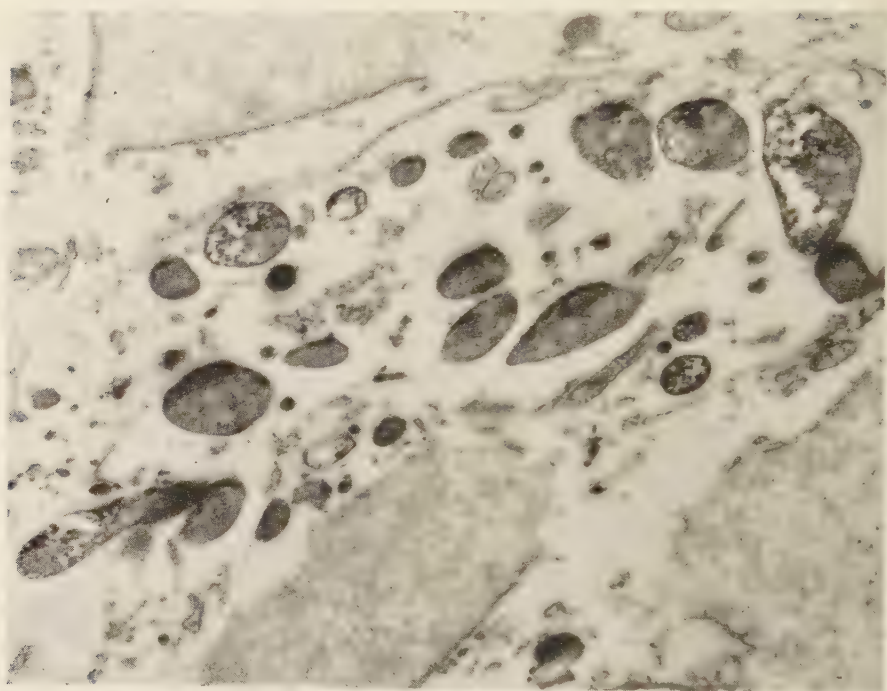


FIGURE 8. Ectodermal cells from chicken embryo chorioallantoic membrane infected with vaccinia virus. $\times 16,000$.

The degeneration of cytoplasmic components other than mitochondria, while presenting a picture no less complex, will be mentioned only briefly. In general, two main types of degeneration are observed: (1) vacuolization and (2) "condensation." Although either type may predominate in a specific cell or even in a system of cells, both are frequently encountered.

Vacuolization. The origin of many vacuoles is not definitely known. Although vesiculation of cisternae and tubules of the endoplasmic reticulum appears to give rise to some, others develop from no evident cytoplasmic component. Generally, swelling and vacuolization of mitochondria add to the number of vacuoles. FIGURE 9, however, illustrates extensive vacuolization without evident mitochondrial disintegration (cells wherein such changes have occurred are generally swollen). The vacuoles vary considerably in size, and several indent the nucleus at the top of the figure. Near the upper and lower borders of the field, only the limiting cellular membrane separates vacuoles from the extracellular space. The presence of an apparently healthy nucleus and numerous intact mitochondria in the interstices raises the question whether these cytoplasmic changes may not be reversible. Cells exhibiting similar vacuolization are frequently found to have ruptured, suggesting that if regeneration does not occur the limiting membrane gives way with a release of cellular contents into the extracellular space.

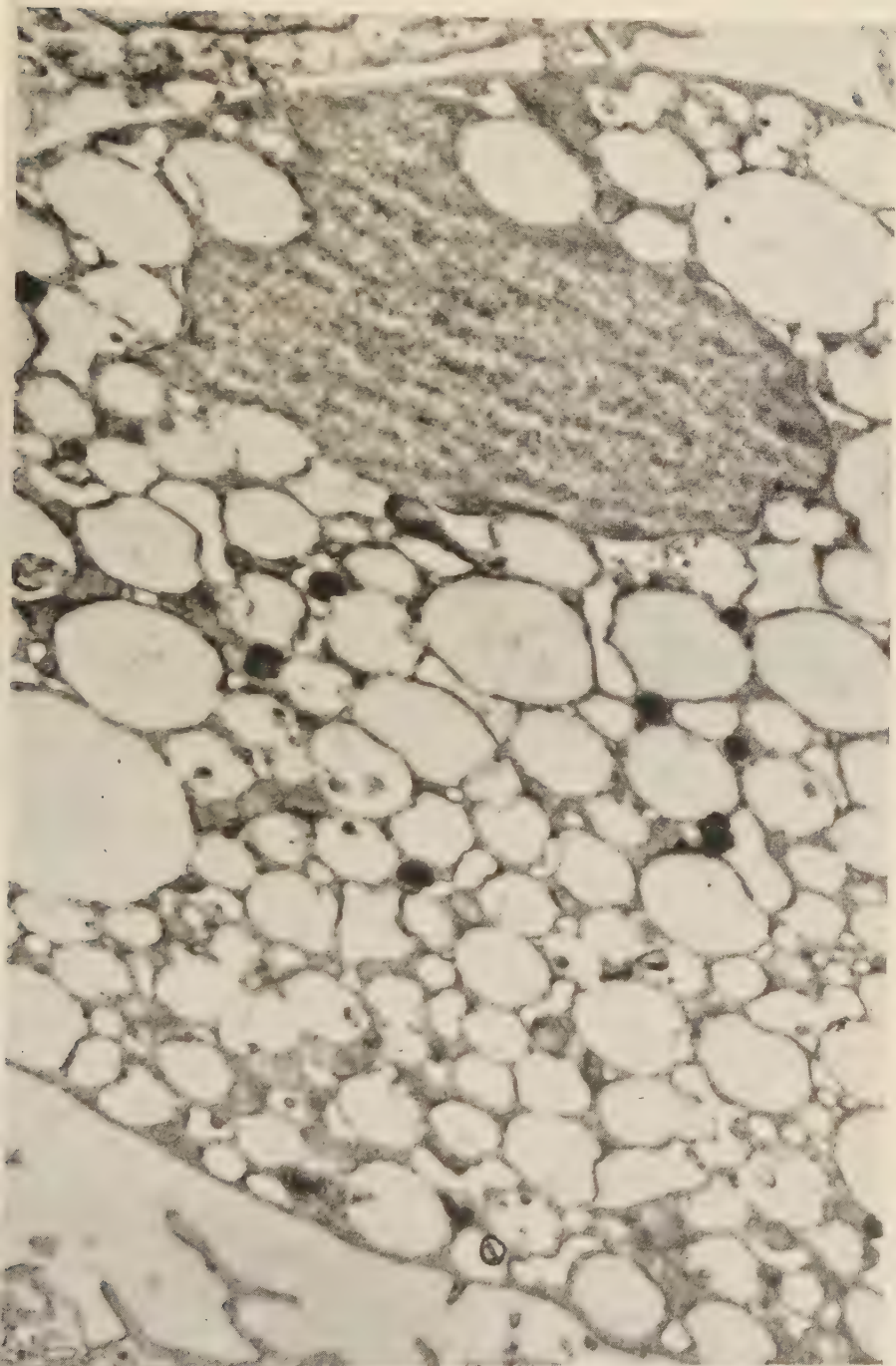


FIGURE 9. HeLa cell from culture infected with adenovirus. $\times 13,000$.

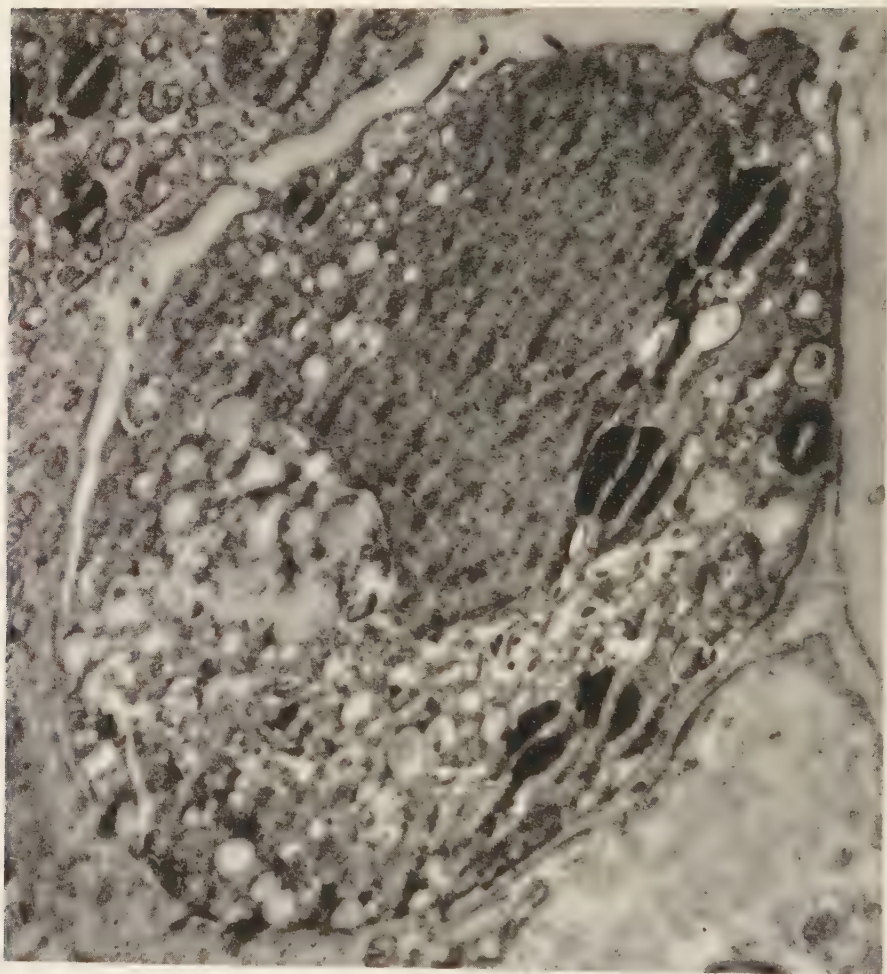


FIGURE 10. HeLa cell from uninfected culture. $\times 11,000$.

Condensation. This process is characterized by cellular shrinkage. The cytoplasm, interspersed with vacuoles and lipid globules, becomes dense and granular, as illustrated by FIGURE 10. The density may reflect shrinkage with resultant packing of preexisting components or of condensation of some new material not normally present; probably both mechanisms operate. Examination of this cell at higher magnification reveals that the mitochondria have disintegrated. The nucleus in the upper half of the field exhibits an irregular outline and contains dense, closely packed granules.

At present the correlation of nuclear changes observed in the light microscope with those encountered in the electron microscope is insufficient to permit detailed analysis. Pyknosis would appear to correspond to the nuclear changes illustrated in FIGURE 11. The shrunken nucleus, although

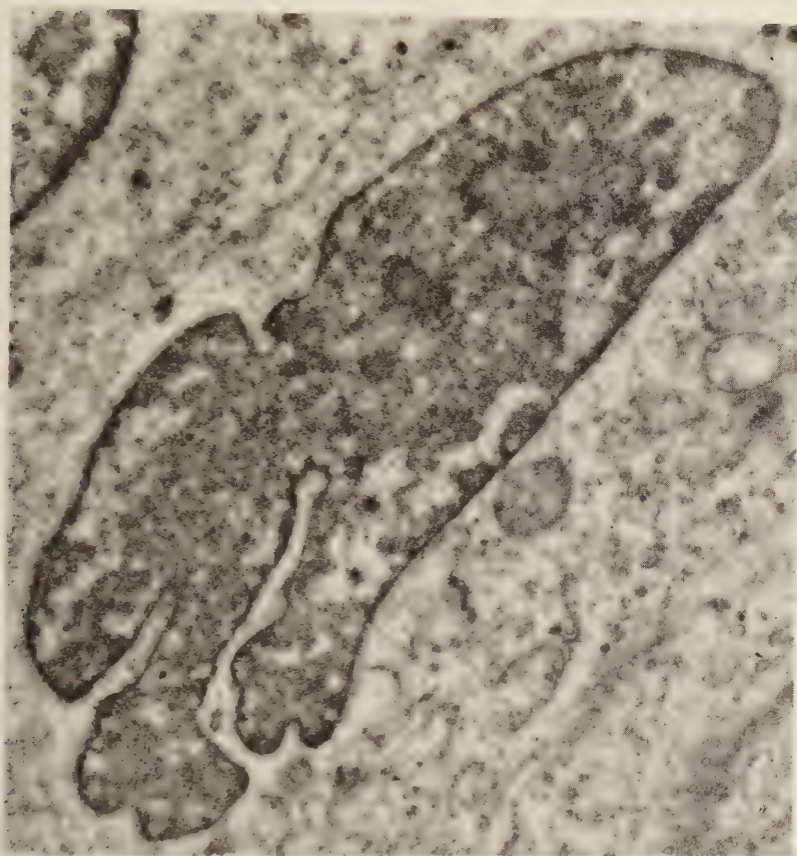


FIGURE 11. HeLa cell from uninfected culture. $\times 13,000$.

still possessing an intact limiting membrane, exhibits an irregular outline and is largely filled by dense granular material. Many of the cytoplasmic components appear to be in the process of disintegration. The nucleus previously shown in FIGURE 10 is probably in a more advanced stage of pyknosis. Fragmentation of nuclei with rupture of the limiting membrane and release of nuclear contents into the cytoplasm is repeatedly encountered and may be equivalent to karyorrhexis. In FIGURE 12, for example, no recognizable membrane separates the nuclear structures occupying much of the middle and lower left third of the field from the cytoplasm. The inset reveals, at higher magnification, the part of the micrograph that has been outlined. A fragment of chromatin may be seen adjacent to two mitochondria. One also encounters nuclei that exhibit a marked decrease in the dense granules constituting the matrix. Margination of chromatin may or may not be present. FIGURES 13, 14, and 15 illustrate characteristic examples. Except at the periphery of the nuclei, the granular components have been replaced by a fine network of threads and small granules of low

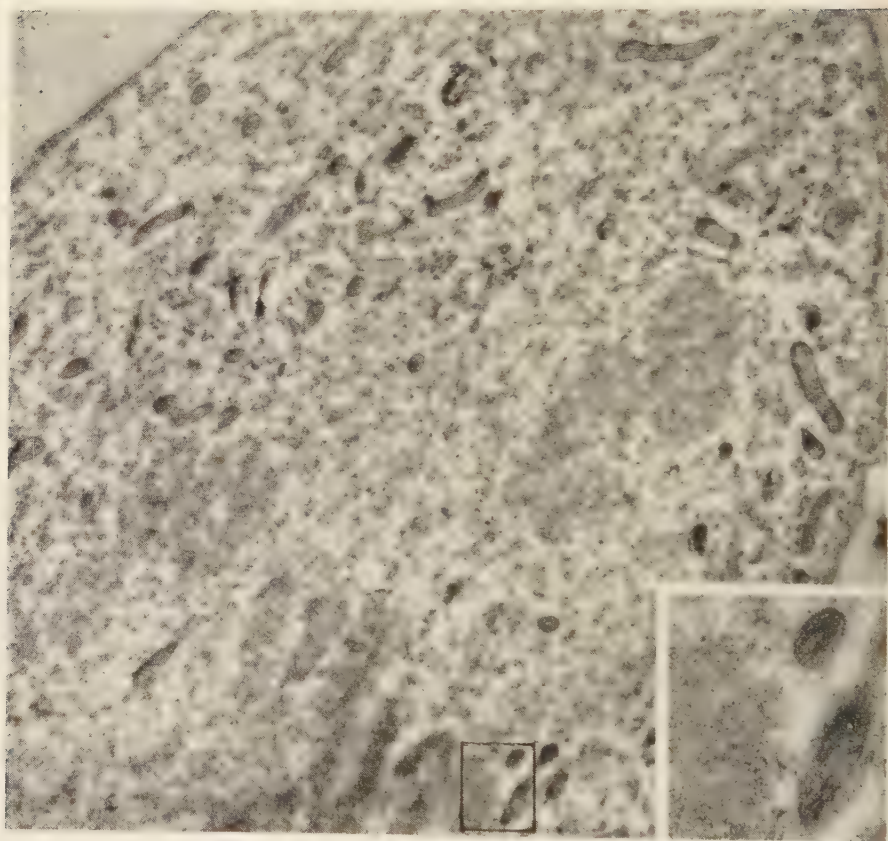


FIGURE 12. HeLa cell from culture infected with adenovirus. $\times 9,300$. Inset $\times 30,000$.

density to the electron beam. It is possible that these changes denote a stage of karyolysis.

Many normal cells exhibit irregular cytoplasmic projections at their free surfaces. During cloudy swelling and necrosis the projections become more numerous. They may be slender or bleblike. FIGURE 16, illustrating the surfaces of two cells, shows both types. At the bottom the extensions are thin, irregular, and frequently branched. Many have been cut obliquely or vertically, and they attach to the cell at a level removed from the plane of this section. At the top the extensions are larger and possess a smooth, rounded contour. When transected at the appropriate level they can be seen to communicate with the cell, suggesting that they form by protrusion of the limiting membrane. Although the majority shown in this field contain only fine granules, it is not uncommon to find extensions possessing a variety of cytoplasmic components.

None of the changes described above is pathognomonic of viral infection, although the frequency of certain changes and the order in which they occur

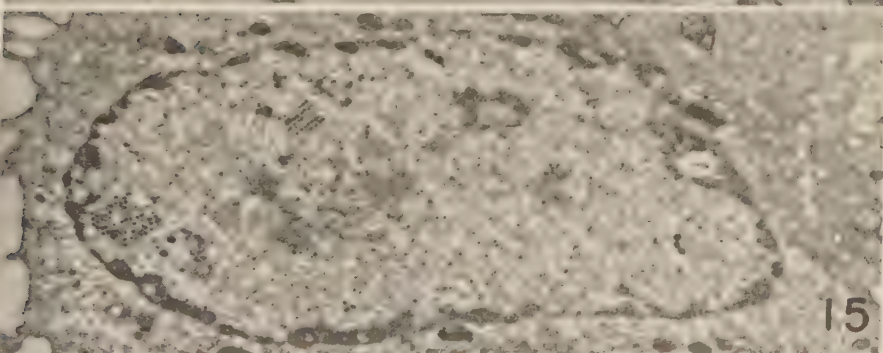
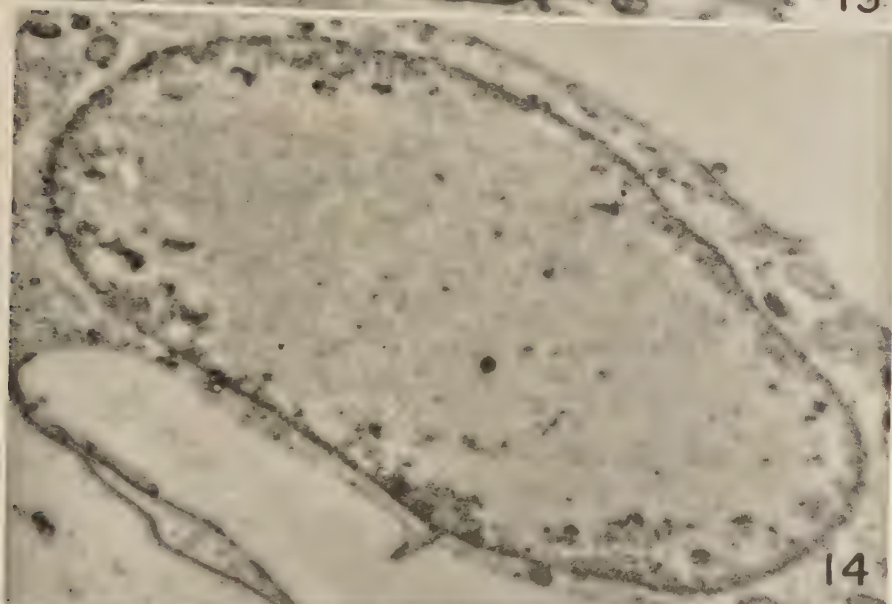
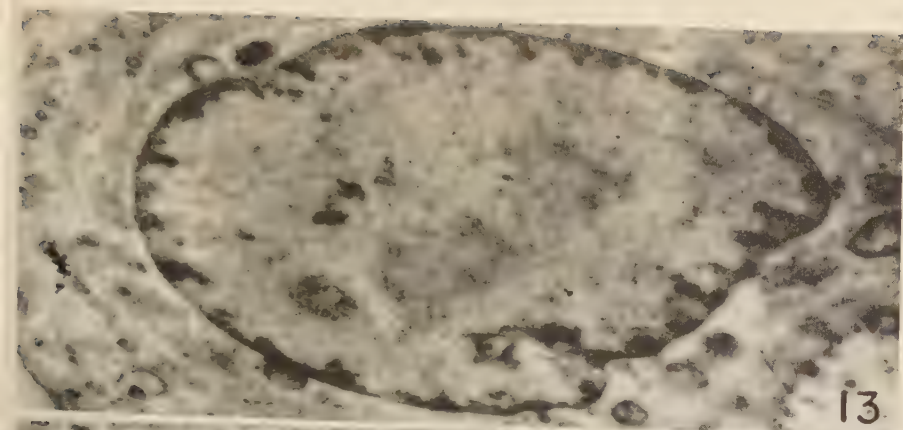


FIGURE 13. Human amnion cell from uninfected culture. $\times 9300$.

FIGURE 14. Entodermal cell of chicken chorioallantoic membrane infected with influenza virus. $\times 14,500$.

FIGURE 15. HeLa cell infected with adenovirus. $\times 9300$.

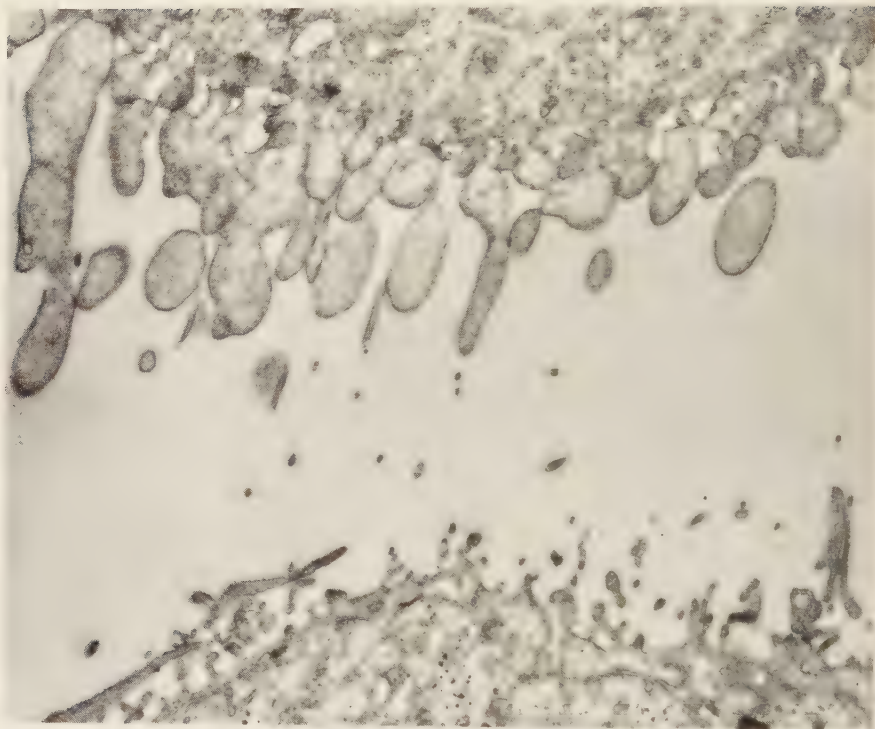


FIGURE 16. HeLa cells from culture infected with adenovirus. $\times 10,000$.

may be suggestive. For example, margination of chromatin is commonly associated with infection by herpes simplex virus²⁶⁻²⁷ or by the adenoviruses*, whereas it is observed only occasionally in nonspecific cellular degeneration†. In addition, these viruses generally produce nuclear changes before there is much cytoplasmic degeneration. In nonspecific necrosis the cytoplasm frequently, but not invariably, exhibits the initial changes. We are faced, however, with the remarkable conclusion that no specific type of degeneration has been demonstrated by electron microscopy to result from viral invasion, multiplication, or release in any mammalian or avian cell system so far examined.

A possible exception may be structures encountered in occasional nuclei of HeLa cells infected with adenoviruses. FIGURE 17 illustrates part of a nucleus containing characteristic viral particles, many of which exhibit a crystalline array. Two aggregates of parallel, dense lamellae are visible. At higher magnification (FIGURE 18) the lamellae, averaging 150 Å. in thickness, appear to consist of poorly defined double membranes. Tubules com-

* Designated as RIAPC viruses by Morgan *et al.*²⁸ in a paper presented prior to the recommendation of a committee on nomenclature, under John F. Enders, that the term "adenovirus" be adopted for this group of infectious agents.²⁹

† Enders and Peebles also noted margination of the chromatin in nonspecific degeneration of cultured monkey kidney cells.³⁰



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FIGURE 17. HeLa cell infected with adenovirus, type 3. $\times 12,000$.

FIGURE 18. HeLa cell infected with adenovirus, type 3. $\times 102,000$.

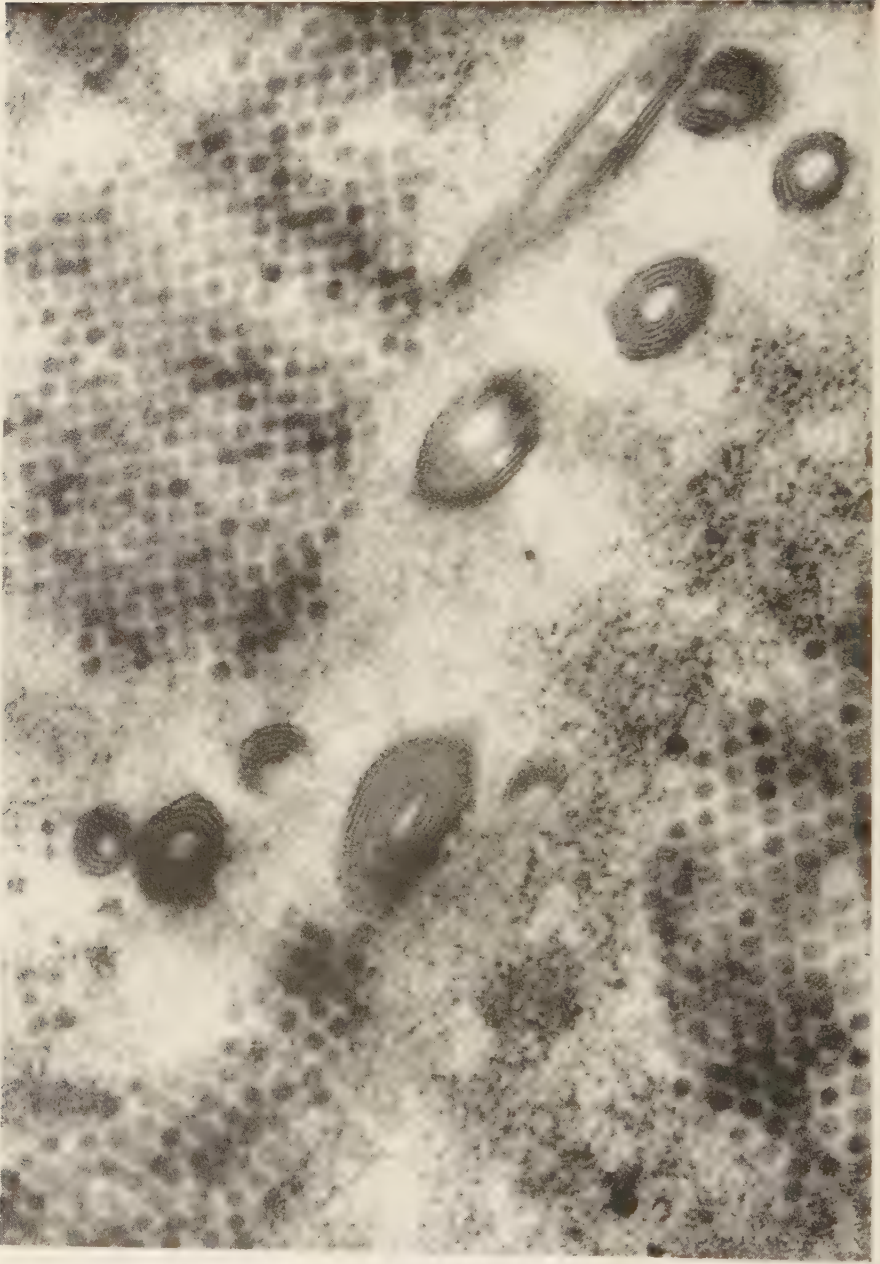


FIGURE 19. HeLa cell infected with adenovirus, type 3. $\times 55,000$.

posed of membranes arranged either concentrically or in spiral form have been observed in some nuclei, as shown in FIGURE 19. In this field they differ in orientation with respect to the plane of section. An obliquely cut tubule near the top of the micrograph contains several viral particles. Three viral crystals are evident in the adjacent nuclear matrix. The tubules and aggregates of lamellae do not show consistent spatial relationships to the virus, nor are they encountered in the majority of infected nuclei. It appears unlikely, therefore, that they are related directly to viral development. They have not been observed in cells showing nonspecific degeneration or in other cells infected with different viruses. Whether they represent a specific nuclear response to adenovirus infection and develop also in other types of cells similarly infected, or whether they are peculiar to HeLa cells and may accompany infection by other types of viruses, remains to be determined.

Viruses Associated with Tumors

Although particles presumed to be virus on the basis of size, shape, or structure have been encountered in association with several types of tumors,³¹⁻⁴² the electron microscope has so far failed to shed any light on the relation of viruses to the pathogenesis of neoplasms. In this connection it is of interest to note that a comparison of vaccinia and fowl pox viruses⁴³ with Shope fibroma virus¹⁵ reveals no differences in morphology and mode of differentiation of these viruses or in the changes they induce in host-cell fine structure. Vaccinia and fowl pox viruses cause initial cellular hyperplasia with subsequent necrosis, whereas the Shope fibroma virus incites a tumor. All three viruses develop within the cytoplasm, where membranes appear to enclose fine granular material. The morphology of each is similar from the early stages, wherein a "nucleoid" and "viroplasm" with a single limiting membrane are evident, to the later stages, characterized by development of a discoid internal body and a double limiting membrane. Bernhard *et al.*¹⁵ called attention to the presence of dense, frequently straight and evenly spaced, parallel lamellae in the cytoplasm of some cells containing the Shope fibroma virus. After publication of their paper, a review of our micrographs revealed similar structures that we had failed to observe in our initial study.¹¹ FIGURE 20 illustrates part of the cytoplasm of an ectodermal cell in a chick chorioallantoic membrane infected with fowl pox virus. Several viral particles are evident. Clusters of dense, nearly parallel membranes exhibit an average spacing of 7 to 10 μ and cannot be distinguished from those encountered by Bernhard and his associates. Although we have observed no morphologic characteristics that permit vaccinia and fowl pox viruses to be differentiated from the Shope fibroma virus, it is evident that a more extensive and detailed comparison of these with other viral agents should be undertaken in the hope of shedding some light on the role viruses may play in initiating neoplastic changes.

Inclusion Bodies

As observed by light microscopy, the tendency of the characteristic inclusion bodies⁴⁴ to develop in mammalian or avian cells as a response to certain

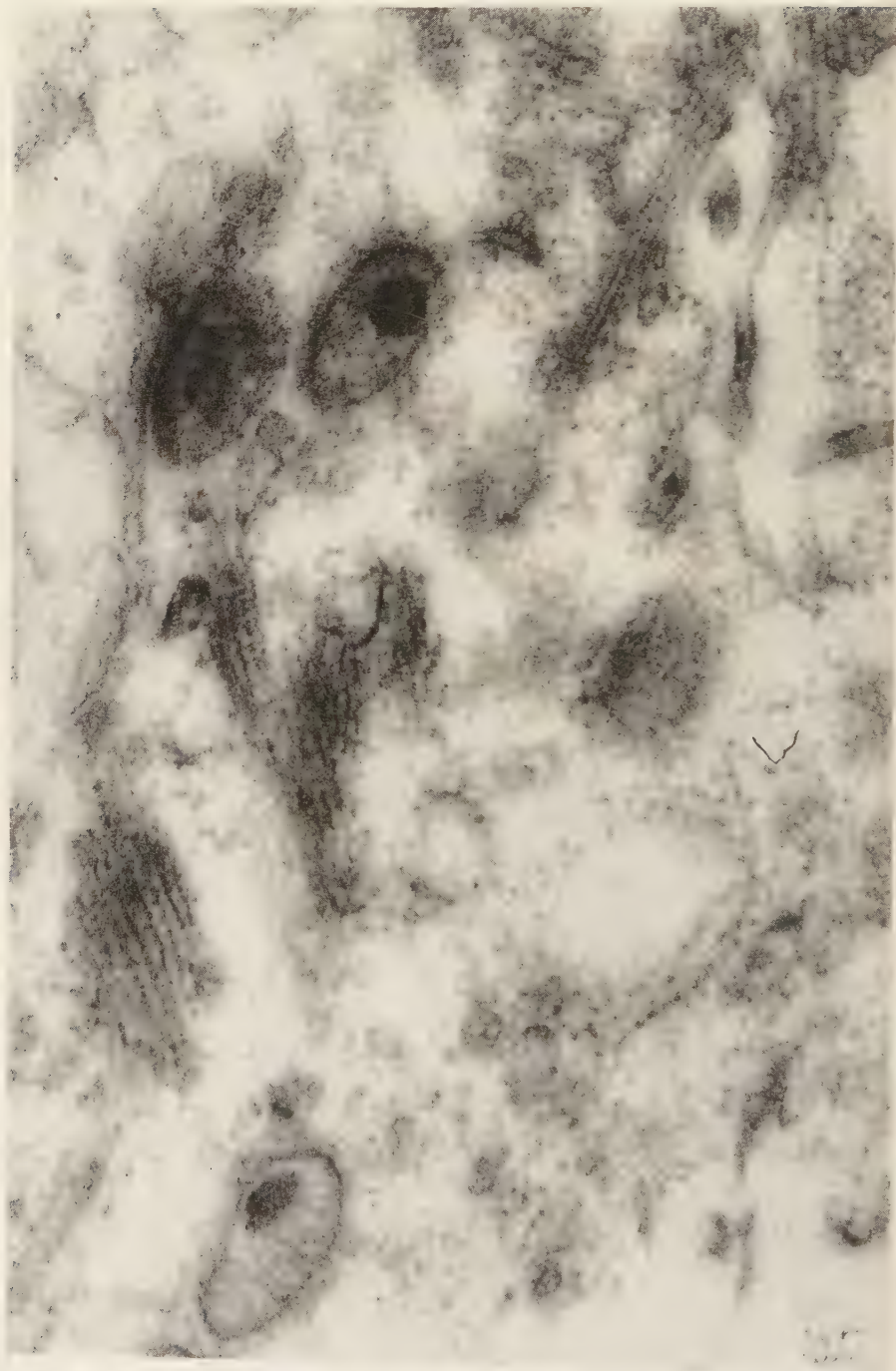


FIGURE 20. Chorioallantoic ectodermal cell infected with fowl pox virus. $\times 86,000$.

viral infections remains unclear. They have been identified largely by their staining characteristics in thick sections. To describe as inclusion bodies structures encountered in unstained sections whose thickness is one one-hundredth of that of sections employed for light microscopy involves a considerable degree of assumption. At present the most suitable method for recognizing inclusion bodies in the electron microscope is the preparation and comparison of contiguous thick and ultrathin sections. Identification of the same structures in the same cells with both electron and light microscopy is then possible. Until the correlative studies of adenoviruses⁴⁵ are extended to include other viruses, little can be gained by speculating on the nature of inclusion bodies and their relation to viral development.

Summary

Distortion caused by polymerization of the embedding plastic has been discussed. Cellular changes encountered in cloudy swelling and necrosis have been described, and a tentative classification has been suggested. It has been emphasized that the degeneration accompanying multiplication of the viruses studied to date is nonspecific. Whether the intranuclear tubules and aggregates of lamellae encountered in HeLa cells infected with adenoviruses represent a specific host-cell response remains to be determined. One virus known to produce a neoplasm (Shope fibroma) cannot be distinguished from vaccinia and fowl pox virus by its morphology, its manner of development, or its effect on cellular fine structure. Much experimental data must be collected and correlated before any real understanding of pathological states in general or of the neoplastic process in particular can be gained through the application of electron microscopy.

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Part II. Effects of Particles on Cells

TRANSDUCTION AND TRANSFORMATION*

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Genetically, transduction and transformation are similar processes: both are the transfer of fragments of the genotype of one cell to other cells. Both processes are important biologically because they can form a cell with a novel genotype. In addition, transduction and transformation can be a method of combining the genotypes of asexually reproducing cells. Both processes are of interest here because both are potentially an origin for neoplastic tissue.

Lederberg (1956b) has proposed that the term transduction be used for these processes in order to emphasize the similarity of the genetic consequences rather than the technical differences in performance. In this paper, transduction will be used as a generic term, and "phage transduction" and "DNA (deoxyribonucleic acid) transduction" will be used in a species sense. The information derived from study of the two processes has been different, however; while both have supplied information about the organization and function of genes, only DNA transduction has provided information on the chemical composition of the gene. This will continue to be the case, at least until the material within phage particles becomes available to manipulation and chemical analysis.

Phage-mediated transduction was first observed in *Salmonella* bacteria by Zinder and Lederberg (1952) and has been extensively studied in this group of microorganisms (Baron *et al.*, 1953; Iseki and Sakai, 1954; Uetake *et al.*, 1955; Zinder, 1954). It was found that a great variety of genetic markers was transduced by phage, including those for amino acid synthesis and carbohydrate fermentation, drug resistance, cell serotype, motility, and others. In fact, because most markers tested have been found to be transducible, it is believed that all the genes of *Salmonella* are subject to transfer by bacteriophage. Markers are usually transduced singly, however, indicating that only small fragments are subject to transfer. Two types of genetic markers can be conferred on a transformed cell by a transducing phage: (1) those that depend merely on the phage for transfer, whose subsequent expression is not dependent on the presence of phage in the clone; and (2) those that are inseparable from the vector of transduction. In the latter group the lysogenized (virus infected) cell is converted to a new geno-

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type, a result comparable to the nontoxigenic to toxigenic conversion obtained by lysogeny in *Corynebacterium diphtheriae* (Groman, 1955). On the basis of these studies it was concluded that bacteriophage was the vector because of similarities in a number of properties between it and the vector of transduction.

The application of transduction as an analytic tool of the genetic material of *Salmonella* cells has been made in several laboratories (Stocker *et al.*, 1953; Demerec, *et al.*, 1955; Hartman, 1955). The results of these studies have shown that (1) some genes of *Salmonella* are sufficiently linked to be transduced simultaneously, and (2) many of these linked genes affect the same phenotype. The first of these findings made possible the mapping of some genes, and the sequences for genes involved in the synthesis of two amino acids, tryptophane and histidine, have been established. Not only was it found that the genes were closely linked, but the gene sequences corresponded to the sequences of enzymatic steps in the respective biosyntheses.

The recipient cell in transduction contains, momentarily at least, two different genes at a single locus; that is, it is heterogenotic for that locus. In the *Salmonella* transduction systems studied, the heterogenotic state does not persist longer than a few cell divisions, at most.

Phage-mediated transductions have been observed also between cells of *Escherichia coli* (Morse *et al.*, 1956a, 1956b; Lennox, 1955; Jacob, 1955) and between the "species" *Escherichia* and *Shigella* (Lennox, 1955). Several different phages were involved in these studies. In general, the frequency of transduction per phage particle was similar to that in *Salmonella*, about 1 per 10^5 to 10^7 . As in *Salmonella*, linked transductions were observed infrequently, but the number of markers transduced at one time was, in some cases, larger than that observed in *Salmonella*. For example, Lennox observed the simultaneous transfer of four "unrelated" genes in *E. coli* K-12 by phage P1. Previously it had been shown that these four markers were linked by bacterial crosses. Both Lennox and Jacob found that the prophage state of one bacteriophage (lambda) could be transduced by another phage. An interesting feature of some of the clones studied by Lennox was the persistence of the transduced fragment which, in one case, was maintained through five serial single-colony transfers of the clone.

The phage lambda-*E. coli* K-12 system studied by Morse *et al.* (1956a, 1956b) has some features that distinguish it from the other phage-transduction systems.

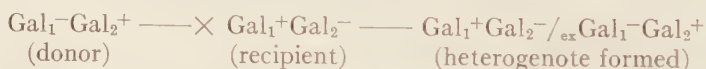
It has been found that lambda, the bacteriophage for which most strains of *E. coli* K-12 are lysogenic, transduces only some genes that affect galactose fermentation (Gal genes). It is to these Gal genes that lambda is found to be linked in bacterial crosses and, presumably, is near (in the prophage form) on the K-12 chromosome. Further evidence that this spatial relationship exists comes from the observation that transducing lambda is found only when produced from lysogenic bacteria; that is, lambda grown from an exogenous source has not been found to possess that transducing activity which lambda from the "Gal-linked" state has.

The number of genes in *E. coli* K-12 affecting galactose fermentation is

large; at least seven have been studied thoroughly, and ten to twenty others less so.

The mutations to inability to ferment galactose that have been deeply studied have been designated as follows: Gal₁⁻, Gal₂⁻, . . . Gal₇⁻ (Morse *et al.*, 1956b). Some of the biochemical steps that these mutations affect have been identified (Kurahashi, 1956). The Gal₂⁻ and Gal₅⁻ mutations result in the loss of galactokinase activity; Gal₁⁻, Gal₄⁻, Gal₆⁻, and Gal₇⁻ mutations cause loss of galactophosphate-uridylyltransferase activity. These mutations have been distinguished as nonallelic by two tests: (1) bacterial crosses and (2) transductional comparisons. In the tests by the crossing method, galactose-positive recombinants were obtained in pairwise crosses between mutants. Tests by the transductional method also gave galactose-positive clones, that is, Gal₁⁻ (donor) × Gal₂⁻ (recipient) yielded galactose positives, but Gal₁⁻ × Gal₁⁻ did not, which also holds true for similar comparisons with the other mutants (Morse *et al.*, 1956b).

Both the positive and negative alleles of the Gal genes have been transduced by lambda. This can be seen most readily from a consideration of an example, the transduction from a Gal₁⁻ donor to a Gal₂⁻ recipient culture. The process may be diagrammed:



The heterogenote formed by lambda transduction, unlike heterogenotes in *Salmonella*, does not segregate to a haploid immediately, but persists and forms a clone that segregates about once per thousand cell divisions. Single gene heterogenotes (—/+), as well as heterogenotes formed between mutants of the kinase group and transferase group, have a galactose-positive phenotype, that is, they ferment galactose. This indicates that under these conditions the positive alleles are dominant to the negative alleles.

The phenotypes of the Gal⁻ mutants and the corresponding single-gene heterogenotes on the indicator medium employed [eosin methylene blue (EMB) galactose agar] are shown in FIGURE 1. The dark colonies are fermenters and the white colonies are nonfermenters. Segregation in the heterogenotic clone is indicated by the presence of nonfermenters and mixed colonies. The segregation process is so rare that heterogenote clones can be maintained by transfer of the positive colonies.

The preparation of the stocks of the Gal⁻ mutants shown in FIGURE 1 will illustrate additional features of the lambda transduction system. In the example given above (Gal₁⁻ × Gal₂⁻), the heterogenote clone formed is galactose-positive and is selected from the large number of nontransformed galactose-negative cells on the indicator medium. With powerful lysates (to be described later) it is possible to make the transductions without selection, or in such a way that galactose-negative clones are produced from galactose-positive recipients. In the latter way the mutations Gal₁⁻ . . . Gal₇⁻ were transduced to the same galactose-positive clone. The stocks with these mutations shown in the figure, with the exception of the Gal genes, are isogenic. The isogenicity is reflected in the similarity of growth of these

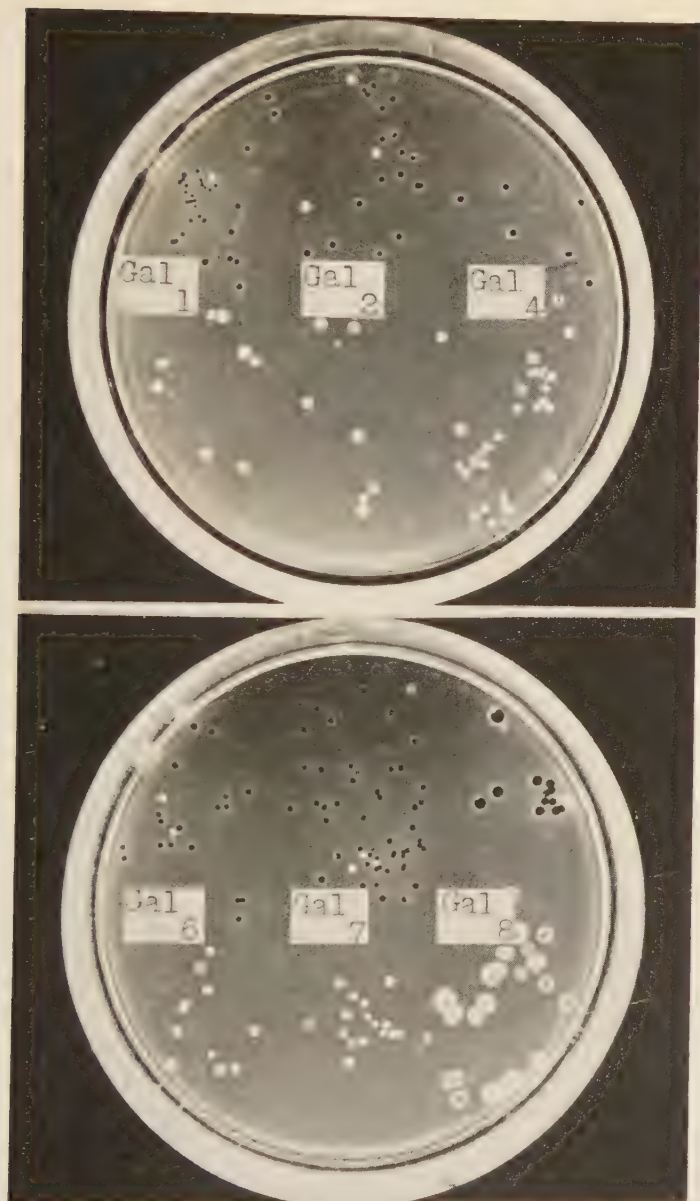


FIGURE 1. The galactose-negative mutants and their respective heterogenotes on EMB galactose agar. The dark colonies are galactose fermenters; the white colonies, galactose nonfermenters. In some cases the colonies are mixed, indicating segregation. The Gal mutants are in the bottom row on each plate, and the corresponding heterogenotes (+/-) are in the top rows. The Gal¹⁻, Gal²⁻, Gal⁴⁻, Gal⁶⁻, and Gal⁷⁻ cultures, with the exception of the Gal genes, are isogenic. Gal⁸⁻ is not isogenic to the other stocks. This is reflected in a difference in colony size from the others. All cultures are prototrophic.

clones. The clone shown with the Gal_s^- mutation was not prepared in this way, is not isogenic with the others, and is reflected in a different growth habit.

As noted, heterogenotes formed between mutants of the kinase group and the transferase group are galactose-positive and resemble the single gene heterogenotes shown in FIGURE 1. Heterogenotes formed between mutants affecting the same enzymatic step (kinase or transferase), with one exception, have a galactose-positive phenotype when the positive alleles are in the *cis* position, but not when in the *trans* position. This indicates a positional effect for these loci. The $++/\text{ex}-$ and $--/\text{ex}++$ *cis* heterogenotes are galactose-positive; the $+ -/\text{ex}-$ and $- +/\text{ex}+$ *trans* heterogenotes are galactose-negative (FIGURE 2). The galactose positives found in the *trans*-position clones are the result of a secondary process, crossing over, that forms the *cis* position ($++/\text{ex}-$ or $--/\text{ex}++$). The *trans*-position clones have been maintained by transferring colonies with a galactose-negative phenotype. Such clones have been shown to contain both alleles at each Gal locus.

The heterogenotes, whether galactose-positive or negative, segregate haploid progeny about once per thousand cell divisions. From a study of many separate segregations from a large number of heterogenotic clones, it has been possible to establish a pattern characteristic of most heterogenotes. The segregants from a heterogenote formed between a Gal^- donor and a nonallelic Gal^- recipient have the following Gal markers: (1) of the recipient cell, about 88 per cent; (2) of the fragment transduced from the donor culture, 10 to 11 per cent; and (3) of the combinations of markers from both donor and recipient, 1 to 2 per cent. This last class includes both galactose positives and double negatives, for example, $\text{Gal}_1^+ \text{Gal}_2^-$ and $\text{Gal}_1^- \text{Gal}_2^-$, respectively. Such combinations indicate that crossing over is taking place in heterogenotic clones.

Further evidence for crossing over is found in the homogenotic segregants (for example, $\text{Gal}_1^- \text{Gal}_1^-$) obtained. Homogenotic segregants are, like the haploids, galactose-negative. Proof that they are homozygous-diploid for the Gal genes is obtained from galactose reversion studies. In contrast with haploid segregants and the parental Gal^- cultures, reversion to Gal^+ changes homogenotes to a heterogenotic condition, and segregation for galactose fermentation is observed. Homogenotic segregants of three types have been obtained from two locus heterogenotes and, presumably, if a sufficiently large sample were studied, the other types could be isolated. The three types that have been observed are: (1) homogenotic for the Gal⁻ mutation of the recipient cell; (2) homogenotic for the Gal⁻ mutation of the donor cell; and (3) homogenotic for one mutation and heterogenotic for the other. The last type of homogenote indicates that crossing over has taken place at the multistrand stage.

Lambda produced from haploid cultures has low transducing activity; for example, 10^8 ultraviolet radiation- (UV-) induced cells yield 10^{10} phage particles, about 10^4 of which have transducing activity. These lysates give a low frequency of transduction with a sample of cells and are called LFT

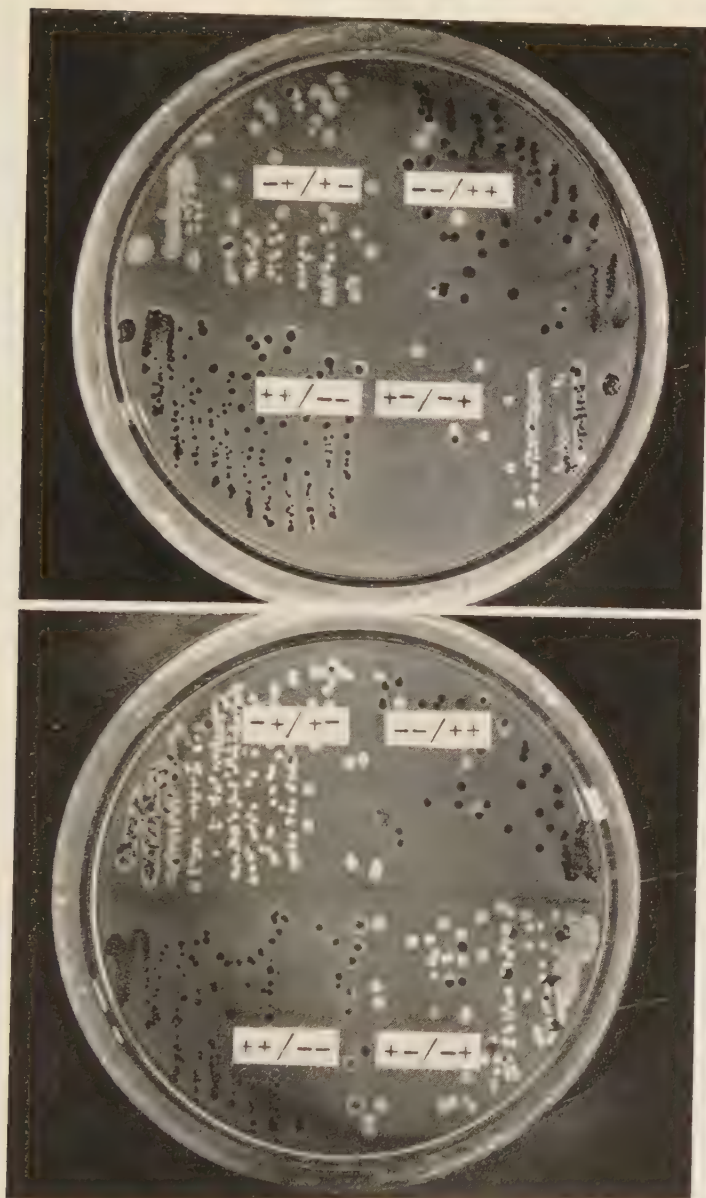


FIGURE 2. Heterogenotes, showing position effect. The formulae indicate the position of the genes; thus, $+ - / - +$ is: genotype of the recipient/genotype of the fragment transduced. In the top plate are Gal_1^- and Gal_4^- heterogenotes; in the bottom plate, Gal_1 and Gal_7^- heterogenotes. In each case the $++/--$, $--/++$ *cis*-position heterogenotes are galactose-positive; the $+--/-+$, $-+/+-$ *trans*-position heterogenotes are galactose-negative. The few galactose-positive colonies in the *trans*-position clones are the result of crossing over during previous growth to give *cis*-position recombinants.

lysates. In contrast with this, UV induction of 10^8 lysogenic (Lp^+), heterogenetic, or homogenetic cells produces about 10^8 lambda particles, nearly every one of which has transducing activity. When undiluted, lysates of the latter type give a high frequency of transduction (1 to 15 per cent of exposed cells) and are referred to as HFT lysates. It is the HFT lysates from homogenetic cultures that are used in Gal typing (Morse *et al.*, 1956b).

The low yield of lambda from heterogenotes and homogenotes suggests that the Gal genes have modified the multiplication of lambda. Further

TABLE 1
PRODUCTION OF TRANSDUCING LAMBDA IN HAPLOID AND HOMOGNETIC CULTURES: RESPREADING EXPERIMENTS

Culture*	Number of cells induced to give transducing particles (spread plates)	Number of transducing particles released per induced cell (respread plates)
Haploid	697	574
	585	719
	704	746
	700	600
Mean	672	658
Homogenote	52	33
	30	35
	32	34
	43	
	50	
	56	
Mean	42	34

The donor cells in saline suspension were irradiated with UV and 0.1-ml. samples were plated on EMB galactose agar. After 2 hr. at 37°C . the indicated plates were respread with 0.1 ml. of broth. Under these conditions, lysis of the cells took place between 60 and 80 min. post UV. See text.

Survival of the UV induced donors in the experiments were: haploid, about 15 per cent; homogenote, 0.5 per cent.

* The transductions were (diagrammatically): haploid, $F^- \text{Gal}_2^- \times F^- \text{Gal}_1^- \text{Gal}_4^- Lp^+$; homogenote, $F^+ \text{Gal}_2^- /_{ex} \text{Gal}_2^- \times F^+ \text{Gal}_1^- Lp^+$.

evidence for this modifying action is obtained from the study of the production of transducing lambda from haploid and heterogenetic and homogenetic cultures and from the study of the transductions produced by lambda from heterogenetic and homogenetic cultures.

The production of transducing lambda was investigated by respreading experiments; that is, a culture was UV-induced to form transducing phage, and samples were spread on a series of plates with an indicator culture. Time was allowed for lysis to take place, and then a number of the plates were respread. Comparison of the respread with the spread plates indicated

whether more than one transducing lambda particle was produced per lysing cell. In experiments with both haploid and heterogenetic cultures, spread and respread plates had equivalent numbers of transductions (TABLE 1). Thus, only one transducing phage was released per cell; the Gal genes had altered the multiplication of lambda from its usual course of yielding about one hundred particles per cell*.

In contrast to LFT transduction, transductions to lambda-sensitive recipients with HFT lysates yielded heterogenetic clones with two phenotypes: (1) those resistant to exogenous lambda and yielding lambda during subsequent growth [such clones being lysogenic (Lp^+) for lambda] and (2) those resistant to exogenous lambda but *not* yielding plaque-forming lambda during subsequent growth. In 58 heterogenotes studied, the relative frequencies of the two types was 45:13, respectively. The first phenotype is explained on the basis of lambda as vector of the transduction. The second can be explained on the basis of a lambda particle modified in some way by its association with the Gal genes. There are two observations that support this supposition: (1) that recipient cells of a mutant type incapable of absorbing lambda do not form heterogenotes of either phenotype and (2) that clones of the second type are resistant to exogenous lambda, suggesting a lambda specificity. One possible explanation is that these heterogenotes are lysogenized with a defective lambda particle, since haploid cultures lysogenized in this manner have been described previously (Lederberg and Lederberg, 1953). The heterogenotes in question, however, differ from lysogenic defectives (in addition to the heterogenetic condition for the Gal genes) in that they have not been found to yield plaque-forming lambda after UV induction, as do lysogenic defectives; also, they segregate lambda-sensitive progeny. On the supposition that these clones are a new type of lambda defective and, on the basis of the last observation, it is possible to give them the tentative genotype $Lp^{r/s}$. A corollary of this supposition is that they are diploid for lambda as well as for Gal genes. A comparison of lambda defectives (Lp^r) with $Lp^{r/s}$ heterogenotes is given in TABLE 2.

When $Lp^{r/s}$ heterogenotes segregate for galactose fermentation, they may segregate for lambda reaction. Thus, of 16 Gal-segregants from a heterogenote in which 2 Gal loci were segregating, 4 were lambda sensitive and 12 were of the parental heterogenote $Lp^{r/s}$ genotype. Gal-reversion studies on these segregants showed that the lambda sensitives were haploid, and the $Lp^{r/s}$ segregants (TABLE 3) were homogenetic (Gal / \times Gal). Other $Lp^{r/s}$ heterogenotes have shown a similar segregational behavior. None have been found to form lambda defectives of the type previously reported in haploids.

If these transductions are the result of defective lambda particles, the defects in lambda are of two types: (1) a defect in the attachment of lambda

* Note (February 20, 1957): Experiments in other laboratories using a different lambda strain and different *E. coli* K-12 mutants have shown that the yield of transducing lambda is greater than one per cell. This indicates that, in some instances, lambda multiplication may not be modified by Gal genes, and also that the Gal genes may multiply as lambda multiplies.

TABLE 2
COMPARISON OF $Lp^{r/s}$ HETEROGENOTES WITH LAMBDA-DEFECTIVE LYSOGENICS (Lp^r)

Cultures	Lambda plaques produced per 5×10^8 cells		Segregate Gal ⁻ and/or lambda- sensitive cells
	Spontaneously	After UV induction	
1. Haploid lambda defectives			
W1924.....	0	2.8×10^4	no
W1027.....	0	7.0×10^2	no
W3172.....	0	1.5×10^4	no
2. $Lp^{r/s}$ heterogenotes			
W2341.....	0	0	yes
371a*.....	0	0	yes
371b*.....	0	0	yes

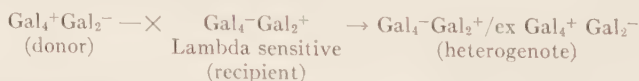
* About 10^6 to 10^7 cells were studied in these cases for lambda output. All $Lp^{r/s}$ segregated lambda-sensitive progeny.

TABLE 3
SEGREGATION FROM AN $Lp^{r/s}$ HETEROGENOTE*

Segregant phenotype	Number of segregants	Gal reversion studies	
		Reversions/segregant	No. reversions segregating
Gal ₂ ⁻ lambda resistant.....	12	1	11
Gal ₂ ⁻ lambda sensitive.....	3	1	0
Gal ₄ ⁻ lambda sensitive.....	1	1	0

The heterogenote was resistant to lysis by exogenous lambda, and it segregated lambda sensitives and galactose negatives. Classification of the segregants is given. The reversion studies indicate that lambda-sensitive segregants are haploid; lambda-resistant segregants, homogenotic.

* The parent galactose-positive heterogenote was made:



to the chromosome of *E. coli*, and (2) a defect such that lambda does not mature to plaque-forming particles.

In summary, it can be said that the association of lambda with the Gal genes has produced modifications of lambda behavior, both in the alteration of the multiplication in the vegetative state, and in the fact that incomplete or defective particles are formed.

DNA-mediated transductions (or transformations) have been described in a variety of bacterial species (Zamenhof, 1956; Ephrussi-Taylor, 1955;

Hotchkiss, 1954, 1956), but most of the investigations have been confined to the *Hemophilus* group and *Streptococcus pneumoniae* (pneumococcus). Although most of the transformations performed were for capsular type and other antigenic properties of the cells, other markers, such as resistance to drugs (streptomycin, penicillin, sulfonamide) and fermentation of carbohydrates (salicin and mannitol), have been transferred via DNA preparations. Most of these markers lack the technical advantages that feature markers employed in the study of phage-mediated transductions, and one of the improvements in DNA transformation studies will be in this area.

As in the phage-mediated transductions, most genetic markers are transferred singly by DNA preparations, but linked transductions have been observed. The frequency of transformation for a given marker under favorable circumstances has been on the order of a few per cent, but in many cases much lower frequencies have been obtained. The many factors that influence the success of transformation will be considered elsewhere in this monograph. The heterogenotes produced by DNA transformation, as in the case of most phage transductions, persist for only a few generations although, in a case of capsule-type transformation in *Hemophilus*, a clone "hybrid" or heterogenetic for two capsular markers was found to persist.

Experimentation with DNA-mediated gene transfers is largely directed towards two goals: (1) to find the minimum amount of DNA required to effect a transformation, and hence the molecular weight of a single gene; and (2) to fractionate DNA into genetic units so that chemical analysis of these units can be made. Some estimates of the required molecular weights have been made; they range from less than one-hundred thousand to several millions. The imprecision here is largely due to technical problems in the biological assay, a subject that will be dealt with by Ravin in the following paper. With regard to the separation of genes in DNA by chemical means, no progress appears to have been made. Apparently, chemical separational procedures have been insufficiently sensitive to accomplish the isolation of genetic units.

With regard to the "prospects for genetics of somatic and tumor cells," attention is called to a paper with this title by Lederberg presented at a conference on ascites tumors held by The New York Academy of Sciences (Lederberg, 1956a).

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THE SIGNIFICANCE OF BACTERIAL TRANSFORMATIONS TO STUDIES OF THE NEOPLASTIC PROCESS*

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The neoplasm has a great deal in common with a clone of mutant cells. While it is possible experimentally to increase the probability of occurrence of a neoplasm by specific kinds of treatment, the neoplastic process also occurs spontaneously at a low frequency. As with mutations, the spontaneous frequency of neoplasms depends upon the genetic constitution of the strain, race, or species with which one is dealing, as well as upon the particular kind of neoplasm. Whether the neoplasm has occurred spontaneously or in response to some specific agent, the cells comprising it appear to be genetically differentiated from the kind of cells from which they originated. In tissue culture, it is possible to grow neoplasms that retain many characteristics that distinguish them from their parental tissues. When transplanted in a new host, after a sojourn in tissue culture, the neoplasm may continue on a course of reproduction, differentiation, and migration characteristic of its type and different from that upon which normal parental tissue would embark following transplantation.¹

Many of the agents that very efficiently induce the neoplastic process are known also to have considerable mutagenic power. Ultraviolet and ionizing radiations, for example, are both carcinogenic and mutagenic.¹ In several species studied,²⁻³ the carcinogenic steroids and nitrogen mustards are also strongly mutagenic. Although we do not understand the mechanisms by which radiation and chemical agents induce mutations and neoplasms, it has been suggested that, within the treated cell, the sites that undergo genetic modification are the same in mutants and neoplasms. Indeed, it might also be expected that, in the spontaneously mutating cell and in the cell spontaneously undergoing the neoplastic process, the site of genetic modification is similar. A somatic mutant and a neoplastic cell would differ, then, only by the particular character under observation: uncontrolled proliferation and, in some instances, migration and invasion are the special characteristics of the neoplastic clone. This is the point of view essentially expressed by the hypothesis that neoplasms are clones of mutated somatic cells.

This hypothesis has had heuristic value, for it has led to an awareness of the importance of the genetic constitution of both neoplasm and host in the genesis and transplantation of tumors.⁴⁻⁵ A direct test of the hypothesis requires, however, a better knowledge than we presently possess of the material that has undergone genetic modification in the neoplastic cell. Fortunately, we have some knowledge of the genetic material within cells and of the localization of cells in organized structures. We know, for example, that mutable genetic material exists in the chromosomes within the nucleus.

* The work reported in this paper is being supported by Grant E 727 of the National Microbiological Institute, Public Health Service, Bethesda, Md.

as well as in such cytoplasmic organelles as plastids,⁶ and in other cytoplasmic particles as yet undetermined morphologically.^{7, 8} We know, too, that nucleic acid is an important specificity-conferring substance present in genetic structures,⁹⁻¹¹ although *in vivo* it is usually associated with protein.

If the mutated genetic material of a neoplastic cell were replaceable by the homologous material from a normal cell, and if the converse were also possible, a means would be available for direct confirmation of the somatic-mutation hypothesis of the origin of neoplasms. For, if the chemical nature and organellar locus of the material being replaced were the same as that involved in mutations in general, the hypothesis would be verified. It becomes important, therefore, to review our knowledge of the ways in which the genetic material of one cell can be replaced with that of another. Here we can turn profitably to an examination of several cases of such "genetic replacements," or "infective heredity," known among bacteria.

At the present time, transfers of genetic material from one bacterium to another are known to be effected by (1) conjugation, (2) transduction, and (3) transformation.

(1) *Conjugation*. This method of transfer between a recipient or host cell and a donor cell involves the "injection" of fragments of genetic material of varying sizes into the host.^{12, 13} Experiments have clearly shown that physical contact is necessary between the host and donor cells,¹⁴ and striking electron micrographs of bacterial cells in the process of conjugation have been made.¹³ The chemical nature of the material injected is not yet known, but it has been demonstrated that the material consists of linked, although separable, units that affect different genetic capacities of the cell.^{12, 13}

(2) *Transduction*. The remaining two processes of "infective heredity" involve a transfer of subcellular units in which direct contact between the donor and host cells is not required. One of these processes, termed transduction, is accomplished by temperate virus particles that introduce portions of the genetic material of their previous host (the donor) into a new host that thereupon incorporates them into its genetic structure.¹⁵ That the genetic material introduced by the virus consists of linked, although separable, units is demonstrable in the same way that linkage and separability are demonstrable in conjugation. Linked units tend to be introduced together, but may be separated as a result of recombination with homologous material in the host.¹⁶ The frequency of recombination is taken as an index of the degree of linkage of the units in question. Suffice it to say that, as in chromosomes of "higher" plants and animals, the linked units can be "mapped" in an orderly, linear array; so that the genetic apparatus of bacteria consists, in part at least, of linkage groups similar to those of more highly evolved organisms.

The work of Hershey and Chase^{10, 17} on the functions of the protein and deoxyribonucleic acid (DNA) components of bacterial viruses has shown that, while the major portion of the protein remains outside, the DNA penetrates the host and apparently directs the synthesis of virus particles as do those initiating the infection. It seems likely, therefore, that DNA is the genetic material introduced by transduction. While it is possible that

much of the transducing DNA is fortuitously combined with viral DNA during the manufacture of mature bacteriophage particles, there is good evidence to indicate that transducing DNA and viral DNA are not wholly distinct materials. The Lederbergs,¹⁸ for example, have shown that a genetic locus concerned with the ability to ferment galactose in the host cell is closely linked to the site of propagation of lambda prophage. Garen and Zinder¹⁹ have offered, moreover, evidence based on radiation studies for at least partial homology between viral and host DNA. Obviously, with respect to that portion of viral DNA that is homologous to host DNA, it would be meaningless to make any distinction between viral and transducing DNA.

Transformation. This third method involves the transfer of DNA,^{9, 20} which is yielded by the donor upon lysis.^{9, 21} The operational distinction between transformation and transduction exists in the resistance of the transducing agent and in the sensitivity of the transforming agent to the enzyme deoxyribonuclease *in vitro*. There is no detectable protein in purified preparations of transforming agents;²² substances that denature or inactivate proteins have no effect on the activity of preparations of transforming agents.²³ Donor cells may autolyze or be induced to lyse by means of surface-active detergents and, provided caution is taken to inactivate the deoxyribonuclease liberated into the medium at the same time, the DNA fraction will transform host cells in the same culture, or the DNA fraction in the medium may be extracted, purified, and used to treat host cells grown under entirely different conditions. Transforming preparations (TP) are quite stable in saline solution and have been kept in an active state for several years.

What are the events that take place when recipient cells are treated with transforming DNA? It is now quite clear that bacterial transformation is a complex process that requires the satisfactory completion of a sequence of stages. The host cells must first become physiologically competent or "sensitized" to react with the transforming DNA.^{24, 25} Although the molecular basis is not known, competence appears to develop cyclically in growing cells,^{26, 27} and is promoted by the presence of albumin in the medium.²⁷⁻²⁹ Once competence arises in the host cells, reaction with the transforming DNA is very rapid; after only a few minutes' exposure of sensitized cells to the DNA, the march of events leading to genetic transformation can no longer be reversed by deoxyribonuclease.^{25, 30} Although there is no appreciable heterogeneity detectable in the genetic capacity of the cells to undergo transformation, at least so far as the pneumococci are concerned,²⁹ the bacteria in a culture do not ordinarily achieve physiological competence simultaneously, and the proportion of competent cells at any given time is small. Thus, when a culture is treated with a high concentration of TP, the number of transformed cells usually constitutes a small fraction of the total population and is not limited by the amount of available active DNA particles. At low concentrations of TP, however, a linear relation holds between the frequency of the cells transformed and the concentration of DNA with which the culture was treated. Typical titration curves are shown in FIGURES 1a and 1b, the latter figure being a magnification

of the region of FIGURE 1a bounded by dotted lines. The linear portion of the titration curve passes through the origin, which is consistent with the assumption that a bacterium reacting with only a single DNA particle will produce transformed progeny.

It has been shown recently that transforming DNA labeled with radioactive phosphorus is picked up by the recipient cells, and that the amount of irreversibly bound DNA is directly proportional to the frequency of the cells transformed.³¹ It seems likely, therefore, that the transforming DNA penetrates the host cell, although the mechanism of its penetration is not well understood. Perhaps the cyclical development of physiological competence in the host cells is associated with their permeability to transforming DNA.²⁷ In any case, it appears that certain species of bacteria have evolved mechanisms of assimilating DNA from the medium. Considering that the probable molecular weight of DNA is over one million,³² this is no mean feat. As a matter of fact, the surface of the host cell probably plays an important role in the uptake of DNA from the medium. Ephrussi-Taylor³³ first noted, for example, that encapsulated strains of pneumococci transform with less regularity than unencapsulated strains. In studies of transformation to streptomycin resistance, I have obtained some quantitative evidence on this point. In TABLE 1 are recorded the frequencies of transformations to streptomycin resistance induced in a variety of strains differing with respect to the amount of Type III polysaccharide contained in the capsule. It will be seen that the activity of a given transforming preparation is highest in the least encapsulated strains and decreases as the amount of capsule increases. It is clear that the transformability of a strain is, in part, determined by the amount of capsular polysaccharide it synthesizes.

In connection with the problem of the mechanism of DNA uptake, it is interesting to note that evidence has recently been reported of transforming DNA that is carried by bacteriophage.³⁴ The transforming activity was destroyed instantly by deoxyribonuclease, although the lytic activity of the preparation was resistant to the enzyme. Furthermore, high-speed centrifugation failed to separate the transforming DNA from the phage particles. It was thus possible in this case, discovered in the *anthracis-cereus* group of the genus *Bacillus*, either that the transforming DNA was fortuitously associated with, but firmly bound to, the outer membranes of the phage particles, thus being exposed to the action of the enzyme deoxyribonuclease, or that the bacteriophage of the *anthracis-cereus* group was permeable to the enzyme. If the latter belief were correct, it would be difficult to understand why the viral DNA, and hence the lytic activity, was not destroyed. If, as seems more likely, the former view is correct, we should have a situation in which a bacteriophage increased the chances for contact or penetration of the host cell by transforming DNA.

In DNA-induced transformations of pneumococci, meningococci, and *Hemophilus*, however, there is no evidence of the existence of a carrier having the kind of morphologic, antigenic, and chemical properties we have come to associate with a virus. In transformations of these species, therefore, DNA alone must be considered. Assuming for the present that trans-

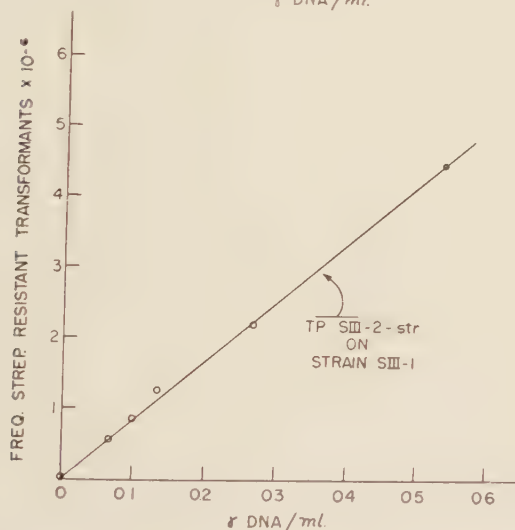
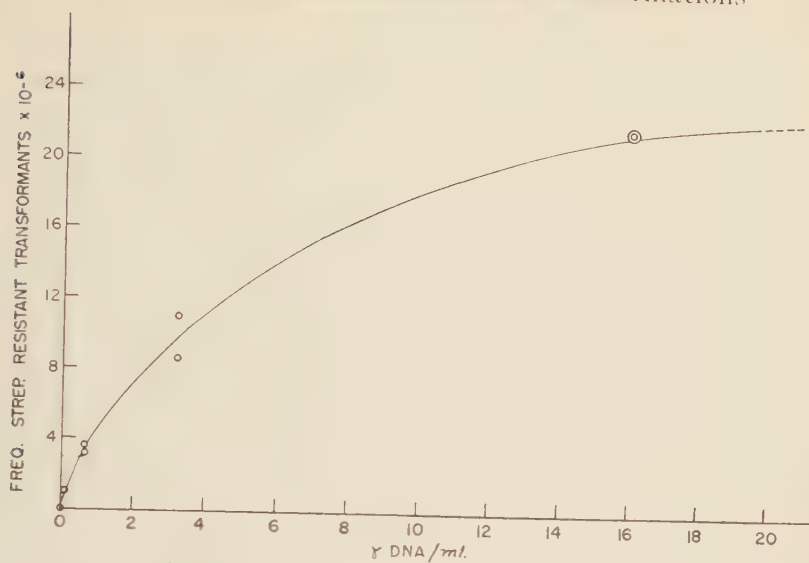


FIGURE 1. FIGURE 1a shows a typical titration curve for transforming DNA. In this case, TP SIII-2-str. was titrated on strain SIII-1. Under the conditions employed in this experiment, physiological competence was attained after 4½ to 6 hr. of growth in the presence of TP; the addition of deoxyribonuclease at this time does not change the eventual frequency of transformants. FIGURE 1b represents the titration of transforming preparation TP SIII-2-str. on strain SIII-1. The transformant frequency/DNA concentration range tested is that indicated by the region bounded by dotted lines in FIGURE 1a.

TABLE 1
MAXIMAL FREQUENCIES OF STREPTOMYCIN-RESISTANT TRANSFORMANTS INDUCED
BY DIFFERENT TRANSFORMING PREPARATIONS ON THREE PNEUMOCOCCAL
STRAINS DIFFERING FROM EACH OTHER IN THE AMOUNT OF TYPE-III
CAPSULAR POLYSACCHARIDE THEY SYNTHESIZE³⁷

Transforming preparations	Experiment No.	Strain		
		SIHI-1 (trace of capsule; rough)	SIHI-2 (encapsulated; nearly smooth)	SIHI-N (fully encapsulated; smooth)
SIHI-1-str.*	226	$\begin{cases} 5.5 \times 10^{-4} \\ 3.6 \times 10^{-4} \end{cases}$	$\begin{cases} 8.1 \times 10^{-6} \\ — \end{cases}$	$\begin{cases} 6.2 \times 10^{-7} \\ 2.4 \times 10^{-6} \end{cases}$
	224	$\begin{cases} 7.2 \times 10^{-4} \\ 5.7 \times 10^{-4} \end{cases}$		
	223		$\begin{cases} 3.0 \times 10^{-6} \\ 2.2 \times 10^{-6} \end{cases}$	
SIHI-2-str.*	226	$\begin{cases} 6.7 \times 10^{-5} \\ 4.8 \times 10^{-5} \end{cases}$	$\begin{cases} 2.5 \times 10^{-6} \\ 4.5 \times 10^{-6} \end{cases}$	$\begin{cases} 1.9 \times 10^{-6} \\ 1.4 \times 10^{-6} \end{cases}$
	224	$\begin{cases} 6.7 \times 10^{-5} \\ 6.9 \times 10^{-5} \end{cases}$		
	223		$\begin{cases} 1.9 \times 10^{-5} \\ 1.9 \times 10^{-5} \end{cases}$	
SIHI-N-str.*	24/VII	$\begin{cases} 3.8 \times 10^{-5} \\ 1.0 \times 10^{-5} \end{cases}$	$\begin{cases} 3.4 \times 10^{-6} \\ — \end{cases}$	$\begin{cases} 2.5 \times 10^{-7} \\ 4.3 \times 10^{-7} \end{cases}$

Conditions established for these experiments were similar to those described in the caption of FIGURE 1. The bracketed figures indicate the results of duplicate experiments.

* DNA preparations extracted from streptomycin-resistant clones of strains SIHI-1, SIHI-2, and SIHI-N, respectively.

forming DNA can and does penetrate these bacteria, we may next ask ourselves what occurs within the host bacterium that has been penetrated by a transforming agent. Does transforming DNA, like the other types of infecting genetic material described, exchange segments with homologous DNA in the host? Or must one regard a transforming preparation as a heterogeneous assortment of DNA molecules, each species affecting a different cellular process? No matter how these specific molecules are organized within the intact cell (for example, as forming the linear structure of a chromosome), the process of lysis and extraction would, according to this idea, effectively separate the different specific DNA molecules. The evidence that bears on this question, unfortunately, does not provide a compelling answer. It is clear that in the host cell there are DNA transforming agents homologous to those extracted from the donor. For example, in the classical case of transformation, pneumococci that synthesize little (or no detectable) capsular polysaccharide were transformed into types that produced a greater amount of capsule.⁹ As we shall see in an experiment to be

TABLE 2
FREQUENCY OF DOUBLE TRANSFORMATIONS AFFECTING THE CAPSULAR
CHARACTER AND RESISTANCE TO STREPTOMYCIN

Transforming preparations	No. cells examined	No. transformants observed	Transformant frequency
I. Transformation of strain SIII-1			
SIII-2-str.	1.5×10^8 6.7×10^7 1.3×10^6	24 SIII-2-str.; 2 SIII-N-str. 4010 Total str. 3 SIII-2-sens.; 3 SIII-N-sens.	Caps.-str. 1.7×10^{-7} Total str. 5.8×10^{-5} Caps.-sens. 4.6×10^{-6}
			$\frac{\text{Caps.-str.}}{\text{Total str.}} = 0.3 \times 10^{-2}$
No transforming preparations	5.9×10^8 1.8×10^6	1 SIII-1-str. 0 SIII-2 or SIII-N	Total str. 1.7×10^{-9} Total caps. $< 1.0 \times 10^{-6}$
II. Transformation of strain SIII-2			
SIII-1-str.	2.0×10^9 2.7×10^8 1.8×10^5	191 SIII-1RT-str.* 2668 Total str. 27 SIII-1RT-sens.*	Caps.-str. 0.95×10^{-7} Total str. 0.98×10^{-5} Caps.-sens. 1.5×10^{-4}
			$\frac{\text{Caps.-str.}}{\text{Total str.}} = 0.9 \times 10^{-2}$
No transforming preparations	5.3×10^8 2.2×10^5	No str. No SIII-1RT*	Total str. $< 1.9 \times 10^{-9}$ Total caps. $< 4.5 \times 10^{-6}$

Saturating concentrations of the transforming preparations were used under conditions described in the caption to FIGURE 1. A number of independent experiments have given, qualitatively, the same results.

Symbols: str., streptomycin resistant; sens., streptomycin sensitive; caps., transformed capsular character.

* A cell type that is less encapsulated than SIII-2, recognized originally by "rough" morphology of the colonies produced.

discussed below, it is also possible to reconvert the transformants with large capsules into pneumococci that produce traces of capsule by utilizing DNA from cells of the latter type (TABLE 2, II). Thus, there is a DNA transforming agent for reduced capsular synthesis as there is one for increased capsular synthesis. Indeed, in every case in which it has been possible to look for reverse transformants, they have been found (filamentous \rightleftharpoons non-filamentous form of growth,³⁵ sensitivity \rightleftharpoons resistance to streptomycin,³⁶ and ability \rightleftharpoons inability to oxidize mannitol³⁶). In other words, in the transformation of a bacterium an endogenous DNA agent is replaced effectively by a homologous exogenous agent. Homologous agents are conceived of, in this view, as alternative forms of a given DNA agent that affects a specific cellular process, analogous to the possible allelic forms of a given gene.

An attractive hypothesis to account for the replacement that occurs in transformation suggests that the transforming agent is a region of a large

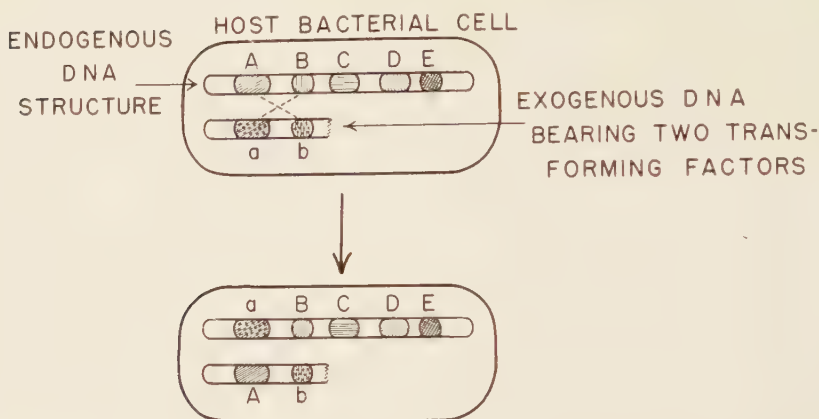


FIGURE 2. Schematic diagram to illustrate the hypothesis that bacterial transformation is a result of the recombination of homologous regions of endogenous and exogenous DNA. The endogenous DNA structure is represented as containing localized regions of specific function (indicated by the different markings) that correspond to the different transforming activities in a DNA preparation obtained from that cell. The exogenous DNA structure is represented as a fragment, although no precise information is available as to the size of the particle that penetrates the host cell.

DNA molecule and that the agent is removed from that molecule by recombination with a homologous site in the endogenous DNA molecule of the host (FIGURE 2). There is good evidence that interactions do occur between exogenous and endogenous DNA. In certain transformations, termed allogenic, a DNA with a novel transforming activity is produced as a result of the confrontation of the host DNA with the donor DNA. It has been shown, for example, that the DNA agent affecting capsular synthesis in pneumococci apparently can be mutated in several ways, each resulting in total or partial loss of the capacity to synthesize capsular polysaccharide.³⁷ When a bacterium bearing one of these agents is treated with the DNA extracted from some other mutant strain, it is often transformed into a fully encapsulated bacterium containing an agent similar to that found in fully encapsulated pneumococci:³³

strain SIII-1 + TP SIII-N \rightarrow SIII-N

strain SIII-1 + TP SIII-1c \rightarrow SIII-N (allogenic transformation)

strain SIII-1 + TP SIII-2 \rightarrow SIII-2 + SIII-N (allogenic transformation)

Ephrussi-Taylor,³³ who discovered the phenomenon of allogenic transformations, has recognized its underlying similarity to recombinations within complex gene loci in "higher" organisms. There arises the question whether all transformations are fundamentally the consequence of recombinations between the host and donor DNA.

Further evidence on this point comes from an analysis of transformations involving a number of different genetic characters. Many such experiments have been done,^{21, 25, 34} and one finds that, in general, the different genetic factors present in a given transforming preparation do not become incorpo-

rated simultaneously. When, for example, one treats a host strain that produces little capsular polysaccharide and is sensitive to streptomycin with the DNA donated by a large-capsule strain that is resistant to streptomycin, one obtains three classes of transformants:²⁵

<i>Host</i>	<i>Donor</i>	<i>Transformant Classes</i>
Reduced capsule + sensitivity to streptomycin	Large capsule + resistance to streptomycin	Large capsule + sensitivity to streptomycin Reduced capsule + resistance to streptomycin Large capsule + resistance to streptomycin

Clearly, then, the factors for resistance to streptomycin and for enhanced capsular synthesis are not incorporated together obligatorily. In this sense, these two factors are separable. Recombination, however, as shown in FIGURE 2, may be the mechanism that separates the different transforming factors.

The important question, therefore, is whether two different transforming factors are incorporated together more frequently than would be expected if they were independent agents. Hotchkiss and Marmur³⁶ were the first to furnish evidence for such nonindependence of incorporation of two different factors. In the case they studied, the factor for mannitol-oxidation and the factor for streptomycin resistance tended to be incorporated together. To a lesser extent, the factor for sulfa resistance tended to be incorporated along with the other two. The factor for penicillin resistance, however, appeared to be independent of the others. In a similar way, I have found that the factor for enhanced capsule production and the factor for streptomycin resistance are incorporated nonrandomly (TABLE 2). The frequency of the class of doubly transformed bacteria—bacteria that are at once encapsulated and resistant—is considerably greater than would be expected on the basis of chance incorporation of two independent agents. On the hypothesis that such nonrandom incorporation of two separable agents is due to physical linkage between the two agents, one could make the prediction that the degree of linkage between two agents will be independent of the specific state of the agents in question (that is, of the allelic form in which the agents happen to be). This prediction has been tested by Hotchkiss and Marmur³⁶ in the case of mannitol-oxidation and streptomycin-resistance transformations, and by myself in the case of capsular and streptomycin-resistance transformations. It will be noted in TABLE 2 that, when a nearly unencapsulated, streptomycin-sensitive strain (SIII-1) is exposed to the TP obtained from an encapsulated, resistant strain (SIII-2-str.), the proportion of encapsulated cells found among the total class of resistant transformants is about three per thousand. When, on the other hand, an encapsulated strain (SIII-2) is exposed to the TP extracted from a nearly unencapsulated, resistant strain (SIII-1-str.), the proportion of resistant transformants that produce less capsule is about the same (about nine per thousand).

It should be pointed out that the capsular transformants (designated as SIII-1-RT) induced by TP SIII-1-str. in strain SIII-2 are not always identi-

cal to SIII-1 cells. Originally they are detected by the striking appearance of the colonies they produce on blood agar; their colonies are definitely "rougher" than those of SIII-2. Upon isolation it is found that many of these transformants differ in some way from the original SIII-1 strain. To be completely agglutinated, all require greater amounts of antisomatic (anti-R) serum than does the SIII-2 strain, a feature usually associated with decreased capsular production.³⁷ However, some of these transformants are more agglutinable with anti-R serum than is the SIII-1 strain; some are less so. All streptomycin-sensitive SIII-1-RT isolates that have been tested, including those more agglutinable than SIII-1, are less transformable to streptomycin resistance with TP SIII-1-str. than is the parent SIII-1 strain (maximal frequency of transformations to streptomycin resistance is at least 20 to 50 times lower than observed with the SIII-1 strain). This finding is not what one would expect if the maximum frequency of transformation were solely a function of the amount of capsule synthesized by a given strain. Work is under way to determine the pattern of transformation reactions that these new (SIII-1-RT) strains will undergo with a variety of transforming preparations, as well as the pattern of transformation reactions that DNA extracted from these strains will induce in a variety of hosts. In this way it is hoped to determine the genotypic relationships of the various mutated SIII strains. At the present time, however, it seems that, on a random basis, the simultaneous introduction of the agent for streptomycin resistance and the agent affecting capsular synthesis occurs more frequently than had been expected.

The hypothesis of linkage can be tested in other ways. For example, the degree of linkage should be independent of the concentration of DNA employed in the experiment. This prediction needs further testing, for, as Ephrussi-Taylor has pointed out,³⁸ linkage has not yet been measured under ideal circumstances, namely, at limiting concentrations of transforming DNA where the probability of contact of a cell by more than one DNA particle is low.

Finally, there is evidence of another kind that bears upon the problem of the linkage of genetic factors on the transforming DNA particle. Schaeffer³⁹ has found that a strain of *Hemophilus influenzae* can be transformed to streptomycin resistance with DNA extracted from a resistant mutant of a strain of the related species, *H. parainfluenzae*. The frequency of this interspecific transformation (heterospecific) is about 10^4 times lower than that of the transformation (homospecific) induced by DNA from a resistant mutant of the *H. influenzae* strain. Except for this difference in frequency, homo- and heterotransformations are indistinguishable (for example, with respect to the time required for development of competence). The interesting finding was then made that the DNA obtained from the streptomycin-resistant cells produced by heterotransformation would transform the strain from which these heterotransformants were derived with the high frequency characteristic of homotransformation. In other words, the frequency of transformation is determined by the kinship of the host strain to the last strain that produced the transforming DNA and, in so doing, apparently

stamped its "mark of origin" upon the DNA. As Schaeffer points out, the facts of heterotransformation suggest that the transforming agent for a given character is not an independent molecule of DNA but, rather, a specific segment in a longer nucleotide chain. The "mark of origin" would involve the segments of the chain adjacent to the specific segment being studied, and these adjacent segments would, in turn, determine to a large extent the frequency of transformation.

The process of transformation does not, in any case, end with the uptake of the transforming agent, regardless of its physical relationship to the DNA particle in the transforming extract. Hotchkiss⁴⁰ has shown that a number of cell generations elapse before the uptake of a DNA agent by a bacterium becomes manifest. Two distinct processes are involved in the delay: (1) the gradual development of the metabolic machinery (phenome) associated with the newly transformed character, and (2) the transmission of the transforming agent, for a number of generations, to only *one* of the daughter cells. The delay in genetic transmission of the agent is obviously a factor contributing to a diminution of the proportion of transformed cells in the culture.

In summary, then, an analysis of bacterial transformation has revealed a considerable complexity of process. Thus, the fact that few bacterial species have been found to undergo DNA-induced transformations (for review, see ⁴¹) is not so surprising as the fact that transformation has been discovered at all. Schaeffer has found, for example, that, under the culture conditions ordinarily employed for *Hemophilus influenzae*, physiological competence appears at a time when cells of this species have nearly exhausted the medium.³⁹ As a consequence, cells that have irreversibly bound the transforming DNA will not express their new character unless good conditions for growth are restored. It remains to be seen whether, with the information now obtained with a few species of transformable bacteria, cases of transformation will be found in other bacterial strains.

This review of our knowledge of infective heredity was undertaken with the view of estimating the feasibility of testing the somatic-mutation theory of the origin of neoplasms. Of the processes described above, transduction and transformation seem most applicable. Conjugation with concomitant "injection" of genetic material may have evolved in certain bacterial species as an adaptive device for promoting genetic recombination. It may be too much to expect that such a phenomenon can or does take place between cells of a multicellular "higher" plant or animal. On the other hand, the susceptibility of many animal and plant cells to viral infection, and the possibility that at least some viruses are mutated descendants of normal intracellular constituents,⁴²⁻⁴³ suggests that transduction may be occurring in multicellular forms in nature. A great stride forward would be made by the discovery of lysogenic tissue cultures.

As a means of inducing genetic modification of neoplastic (or normal) cells of higher organisms, however, treatment with appropriate DNA extracts would appear particularly promising. At the outset we should face one difficulty inherent in the study of mutations in diploid or polyploid material, in that the introduction of recessive alleles would be masked. Therefore,

the possibility of using dominant markers, or markers for which the cell is hemizygous, would necessarily be considered. Information obtained from studies of bacterial transformation, however, indicates that other pitfalls could be anticipated. First, the surface properties of the cells chosen to serve as hosts for the transforming DNA would require careful consideration. Cells that secrete considerable gummy or adhesive materials might be impenetrable to DNA. Furthermore, it would be necessary to avoid cells that secrete large amounts of deoxyribonuclease or, alternatively, we should add inactivating agents of the enzyme to the medium.⁴⁴ Second, the greater the genetic dissimilarity between the host and donor cell types, the less would be the chance for successful incorporation of DNA in the host. In this connection, King and Briggs⁴⁵ have obtained results of nuclear transplantation experiments that they interpret as indicating that the nuclei of the differentiating cells of the amphibian embryo undergo genetic changes. Proof of stable nuclear changes in developing somatic cells obviously would have a great bearing on the problem of neoplastic growth. Third, the delay in genetic transmission of the transforming agent may be a function of the number of gene strands per chromosome in the host cell. Assuming that transformation is potentially a nuclear phenomenon in cells of higher organisms (since DNA is found to the greatest extent in chromosomes), and assuming, further, that the transforming agent is incorporated into one of the gene strands of which the chromosome is composed, then the appearance of a stable clone of transformed cells will be delayed to an extent depending on the number of homologues from which the transforming agent will necessarily be segregated during cellular reproduction.

Finally, of course, it must be re-emphasized that neoplastic cells of different kinds may have different origins, so that, conceivably, mutations in the DNA of genetic structures may not be the basis of all neoplastic growths. In spite of all the foreseeable difficulties in the way of analyzing the genetic basis of neoplastic growth, it may be hoped that the information already gained about infective heredity in microorganisms will prove useful to the future geneticists of somatic and tumor cells.⁴⁶

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A TUMOR-INDUCING FACTOR IN *DROSOPHILA MELANOGASTER*. I. PURIFICATION AND ACTION*

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During the past forty years considerable research has been concerned with the formation of melanotic tumors in *Drosophila melanogaster*. Some investigations have associated the formation of these tumors with tumor genes, while others have determined the effects of environmental modifications upon their incidence. Two phases of the tumor problem in *Drosophila*—tumor etiology and host susceptibility—have been conspicuous by their absence from the literature.

The etiology of melanotic tumors in *Drosophila* remained enigmatic until it was reported that tumor formation in the *tu-e* strain is associated with an etiological factor present in acellular *tu-e* extracts.¹ The presence of an active tumor-inducing factor (TIF) was determined by the injection of aliquots of the *tu-e* extract into larval hosts of the *wild 51-52* strain.¹ This host strain is a tumor-free, genetically stable, and hardy strain from which no active tumor-inducing factor can be extracted.¹⁻³ Nevertheless, *wild 51-52* hosts are highly reactive to the injected TIF. Recent investigations have demonstrated that, under the proper conditions, active tumor-inducing factors may be extracted from all tumor strains of *Drosophila melanogaster*.

The tumor-inducing factor and its relation to tumor formation in the *tu-e* strain of *Drosophila melanogaster* is being investigated. The *tu-e* TIF, when injected into *wild 51-52* hosts, induces the formation of growths that are histologically similar to those present in the *tu-e* donors.⁵ Repeated tests, using refined injection techniques and followed by histological examinations, have eliminated the possibility that injury is the causal factor in the induction of tumor formation in the *wild 51-52* hosts. The refinements in injection techniques are (1) the insertion of the needle toward the posterior region of the animal, at the tenth to twelfth body segment, and (2) the fact that the needle, inserted almost parallel to the exoskeleton, is never permitted to approach any of the deeper vital organs. The activity of the *tu-e* TIF increases with the age of the larval donors and is not demonstrable in extracts of donor pupae or adults.⁶ It is significant that no active TIF is present in *tu-e* extracts,¹ and spontaneous tumor incidence in the *tu-e* strain is drastically reduced when *tu-e* animals are reared on a nutritionally deficient diet.

Purification Process

For convenience in evaluating TIF activity in solutions with an unknown amount of the factor, an assay method was devised.³ By the application

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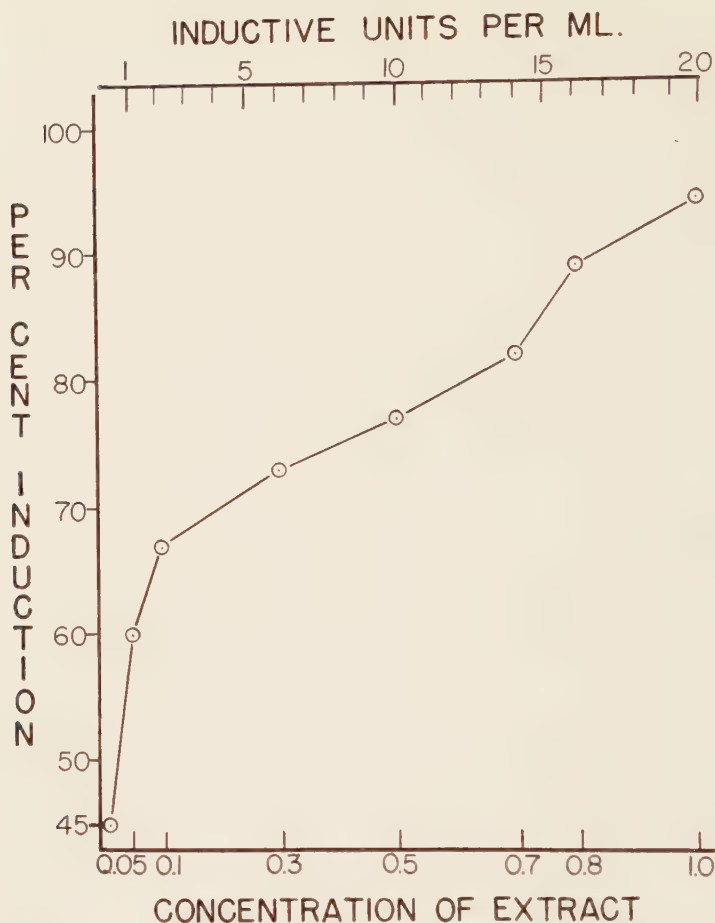


FIGURE 1. Assay curve for the determination of the tumor-inducing factor.

of this method, the number of inductive units per milliliter of TIF preparation can be determined (FIGURE 1). This assay, in conjunction with the protein-nitrogen content of TIF preparations, was used in the evaluation of the steps in the purification process.

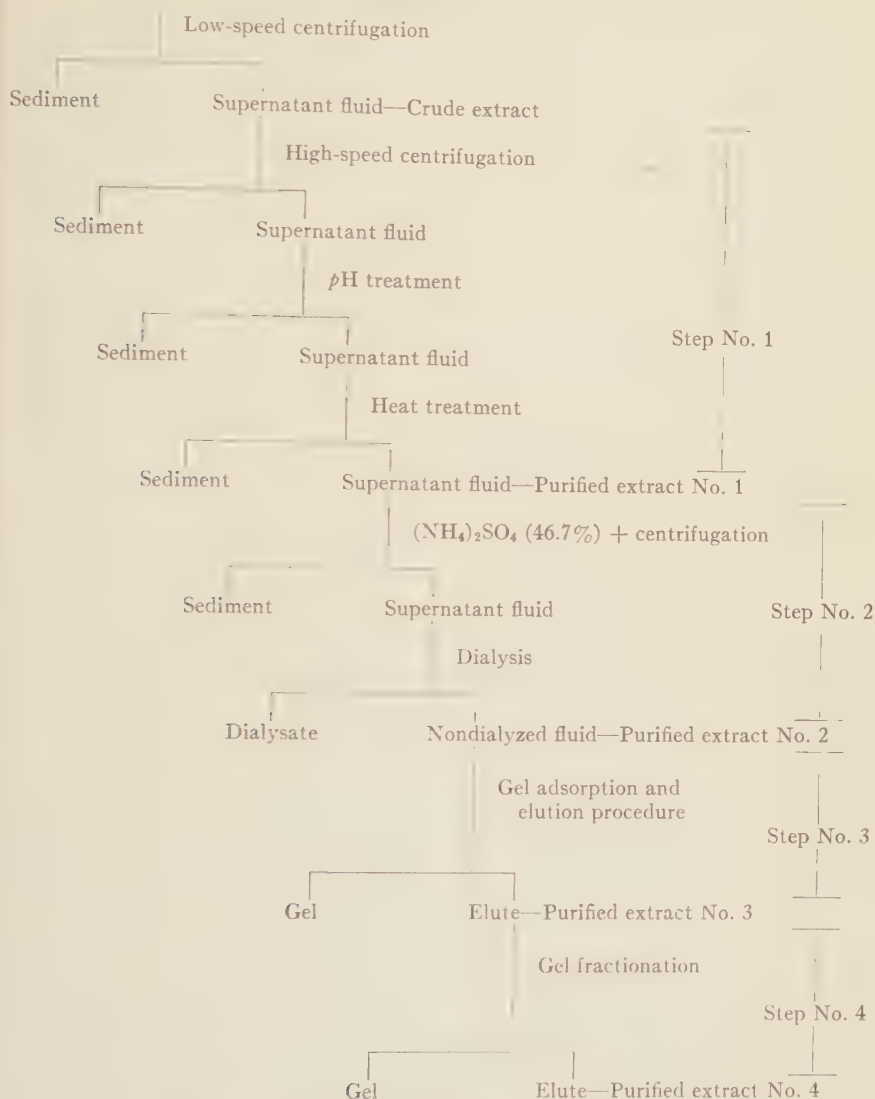
The process used for the purification of crude TIF preparations consists of 4 major steps (TABLE 1).

Crude extracts. An 88.8-gm. sample of fresh 96-hr. *tu-c* larvae, ground in a mortar and suspended in 200 ml. of "Tris" buffer (0.05 *M* at pH 7.8), was centrifuged at $6500 \times g$ for 15 min. The supernatant fluid (crude extract—250 ml.) contained 425.8 mg. of protein nitrogen. One ml. of crude extract contained 20 inductive units (TABLE 2).

Step No. 1. The crude extract was centrifuged at $35,000 \times g$ for 15 min. at $0^{\circ} C$. Subsequently the supernatant fluid was adjusted to pH 5.0 and allowed to stand for 5 min. After centrifugation at $98,000 \times g$ for 5 min.,

TABLE 1
PURIFICATION PROCESS OF THE TUMOR-INDUCING FACTOR

88.8 gm. mashed larvae + 200 ml. Tris buffer



this supernatant fluid was readjusted to pH 7.0 and exposed to a heat treatment of 55° C. for 15 min. After cooling to 25° C. and centrifugation at $9800 \times g$ for 5 min., the supernatant fluid (850 ml., purified No. 1) contained 325 mg. of protein nitrogen. In order to eliminate the effects of dilution upon TIF activity, 3.4 ml. of purified No. 1 was reduced to 1 ml. and assayed (1.0 inductive unit per ml.), as shown in TABLE 2.

TABLE 2
RESULTS OF PURIFICATION PROCESS AS DETERMINED BY ASSAY
AND PROTEIN NITROGEN CONTENT

	Crude extract	Purified No. 1	Purified No. 2	Purified No. 3*	Purified No. 4
Hosts.....	325	442	528	1300	928
Emerged adults.....	95	111	95	311	161
Per cent survival.....	26	23	18	24	17
Tumorous adults.....	89	67	87	257	128
Per cent induction.....	94	60	92	83	80
Inductive units per ml.....	20	0.3	2.8	140	254
Volume in ml.....	250	850	1600	25	12
Total inductive units.....	5000	255	4480	3500	3046
Total protein nitrogen.....	426	325	36.5	1.44	0.022*
Inductive units per mg. protein nitrogen	12	0.8	122	2430	138500
Purification.....	—	—	11X	208X	11800X

* Since absolute accuracy could not be attained in the determination of this small amount of protein nitrogen, this figure is a mean of many samples.

Step No. 2. Purified extract No. 1 was brought to 46.7 per cent saturation (at 25° C.) with ammonium sulfate. After standing for 10 min. and subjecting it to centrifugation at $9800 \times g$ for 5 min., the supernatant fluid was dialyzed against 4 liters of Tris buffer at 4° C. for 16 hr. The fluid remaining in the dialysis bags (purified No. 2, volume 1600 ml.) contained 36.5 mg. of protein nitrogen. To eliminate the effects of dilution upon TIF activity, 6.4 ml. of purified No. 2 was reduced to 1 ml. and assayed (18 inductive units per ml.). The procedures used in this step apparently purified TIF eighteenfold (TABLE 2).

Step No. 3. Purified No. 2 (1600 ml.) was divided into 40 aliquots. Each 40-ml. aliquot (adjusted to pH 6.5) was treated with 1.5 ml. of calcium phosphate gel. After 10 min., this mixture was centrifuged at 5000 g for 2 min., the supernatant fluid was discarded, and 5 ml. of Tris was added to the gel. After 1 hr., each of the 40 aliquots was centrifuged at $5000 \times g$ for 2 min. The volume of the combined supernates (200 ml.) was divided into 40 aliquots, and each was treated, as previously described, with 1.5 ml. of phosphate gel. The final supernatant fluid (25 ml. purified No. 3) contained 1.44 mg. of protein nitrogen. To eliminate the effects of overconcentration upon TIF activity, 0.1 ml. of purified No. 3 was diluted tenfold and assayed (14 inductive units per ml.). TIF was purified two hundred and eightfold by the procedures utilized in step No. 3 (TABLE 2).

The 25 ml. of purified No. 3, adjusted to pH 6.5, was treated with 1 ml. of calcium phosphate gel and agitated on a mechanical shaker for 10 min. After centrifugation at $5000 \times g$ for 2 min., the supernatant fluid was discarded and the gel was washed with 3 ml. of 0.25 M solution of Tris buffer (adjusted to pH 6.5). The washed gel was mixed with 3 ml. of 0.01 M Tris buffer (adjusted to pH 7.5). At the end of 10 min. of agitation the mixture was centrifuged at $5000 \times g$ for 2 min. and the supernatant fluid was dis-

carded. The addition of 3 ml. of 0.01 *M* Tris buffer at pH 7.5, agitation, centrifugation, and disposal of the supernatant fluid was repeated. Thereafter, in 4 sequential steps, 3 ml. of 0.05 *M* Tris buffer at pH 7.5 was added to the gel. After each agitation for 10 min., the mixture was centrifuged at $5000 \times g$ for 2 min. The 4 combined supernates (12 ml.) constituted purified extract No. 4. To eliminate the effects of overconcentration upon TIF activity, 0.1 ml. of purified No. 4 was diluted with 20.7 ml. of Tris buffer (0.05 *M* at pH 7.8) and assayed (12 inductive units per ml.). Thus, the procedures used in steps 1 to 4 purified TIF approximately twelve thousandfold (TABLE 2).

Modifiers of TIF

Inactivator. The activity of TIF could not be maintained in crude extracts stored at -20°C . Since TIF activity could be maintained in purified extracts (stored at -20°C), the procedures utilized in step No. 1 removed and/or inactivated some factors that were preventing the maintenance of TIF activity in crude extracts.

Inhibitors of TIF activity. In addition to materials that affect storage of TIF, the data indicated that crude extracts and purified fraction No. 1 contained substances that inhibited the activity of TIF. The low activity of fraction No. 1 could be accounted for by the presence of inhibitory substances that were perhaps concentrated or activated in step No. 1 and removed in step No. 2.

This hypothesis receives support from the results of experiments in which crude preparations of the inhibitors were obtained by the following procedure: the sediments produced in purification step No. 2 (by the centrifugation at $9800 \times g$ for 5 min. of TIF-ammonium sulfate mixtures) were resuspended and dialyzed against Tris buffer for 16 hr. Subsequent tests indicated that the inhibitors were nondialyzable. A sample of purified TIF (4 inductive units per ml.) was added to an aliquot of the crude preparation of inhibitors. After 30 min. of agitation and subsequent standing for 30 min., these mixtures were assayed for TIF activity. These assays indicated that the activity of the 4 inductive units per ml. of purified TIF was absent.

Modifier of TIF action. The tumors induced by TIF in crude, purified No. 1 and No. 2 preparations were usually free-floating, not associated with normal tissue, and located in the posterior portion of the animal (caudal hemocoel).⁵ These characteristics and the disposition of the tumors are similar to the usually benign tumors found in the *tu-e* donors. In contrast, the tumors induced in host animals by TIF in purified preparations No. 3 and No. 4 were attached to and associated with normal tissues and invariably were disposed anteriorly.⁵ Moreover, it was observed that most of the hosts that had been injected with the *crude* preparations of TIF, and had not reached adulthood, were not tumorous, whereas most of the hosts that had been injected with *purified* TIF (Nos. 3 and 4), and had not reached adulthood, had died with large tumors in the anterior portion of the body. Due to these differences in the characteristics and the disposition of the tumors induced by the TIF in crude and purified preparations, the existence of a "modifier" of tumor-inducing-factor action was inferred.³

Histology and Tissue Culture of Induced Tumors

Since possible differences between tumors induced by TIF in crude and purified preparations were observed, a histological study was conducted to elucidate some aspects of these differences.⁵

Approximately three to seven days after they had been injected with TIF preparations, all hosts (live and dead) were prepared for histological study. All induced tumors were histologically similar to those present in the *tue* donors. The basic cellular component of these tumors is composed of spindle-shaped cells. Cells of this type have never been observed in histological sections of animals of the *wild 51-52* host strain, nor have these atypical cells and/or tumors been observed in *wild 51-52* hosts that had been injured and/or injected with diluting medium or larval extracts of tumor-free strains.

Histological sections of hosts injected with crude preparations of TIF indicated that the posteriorly disposed tumors were rarely associated with normal tissue. The benign nature of these tumors is indicated by the fact that most of the hosts, with these induced growths, can pupate and reach adulthood without apparent adverse effects. The hosts injected with purified preparations (Nos. 3 and 4) of TIF had an abnormally high mortality rate. Histological sections of most of these injected hosts, which died before pupation with large tumors in the anterior portion of the animal, revealed that the tumors were associated with normal tissue and, in some cases, had infiltrated and apparently destroyed such tissue. Some tumors were associated with imaginal tissue while, in other instances, tumor cells had been found in pharyngeal muscle. Areas of this muscle were indefinable and were seemingly replaced by tumorous tissue. It was also observed that tumors had been associated with parts of the brain, gut, and fat body; tumor cells had infiltrated into gut and fat body.⁵ Moreover, it was apparent that parts of the outer membrane of the brain had been destroyed by invading tumor cells. In still other animals, tumors were associated with larval mouth parts, esophagus, and body-wall muscle.

The possibility existed that tumor formation and the infiltration of tissue is a result of the association of leukocytes with previously damaged tissue (caused by injection trauma). This explanation for the induction of invasive tumors is not considered valid because (1) at no time is the site of injection nearer than seven body segments from the anteriorly disposed tumor site; (2) histological examination indicated that no tumor or tumor cells were associated with the injection site; (3) histological examination failed to reveal the presence of tumors or tumor cells in animals injured or injected with fluids other than tumor-inducing-factor preparations; and (4) there was no evidence to demonstrate that normal tissue (other than body wall) had been injured by injection.

The tumors induced by purified TIF were intimately associated with normal tissues and had apparently invaded and destroyed tissues of vital structures. Consequently, the high mortality rate of these hosts could be linked to the presence of these invasive and destructive tumors. This observation gave credence to the suggestion that there were differences between the tumors induced by TIF in crude and purified preparations. It has been sug-

gested that the underlying cause for these differences may be that modifiers, inhibiting the action of the TIF in crude preparations, are not actively present in purified preparations. Therefore, after the removal of the action modifiers, the *tu-e* TIF acquires the potentiality of inducing invasive tumors. Thus, it is possible that the benign nature of the *tu-e* spontaneous tumors, tumors induced by TIF in crude preparations, as well as the "pseudotumors" described in other strains of *Drosophila* may be the result of the inhibitory action of comparable modifiers upon tumor-inducing factors.

Using a new culture medium,⁵ hanging-drop cultures of induced tumors were prepared. Two types of induced tumors were cultured, namely, those affiliated with tissue and those that appeared to be "inert" black bodies. After fourteen days of incubation, tumor cells had infiltrated adjacent normal tissue in the explant. In various areas there were dense groups of tumor cells that subsequently became more pigmented. The pattern of pigmentation appeared to be fibrillar.

After a similar incubation period, cells appeared at the surface of the "seemingly inert" tumor. Some of these cells migrated away from the tumor, while others remained at the surface. These cells were stellate in shape and had fine cytoplasmic dendrils. Pigment granules were concentrated around the nucleus, but were finely dispersed throughout the cytoplasmic extensions.

The appearance of cells in the culture of the inert, black bodies appeared significant. In the literature, such tumors have been called "clumps of dead melanized cells," or "amorphous, friable black masses." The appearance of free and attached tumor cells in the tumor culture indicated that there were areas of living tumor tissue in these seemingly inert black masses. Mitosis could have occurred but, under these culture conditions, conclusive evidence of this activity was not obtained.

Summary

It is suggested that the formation of melanotic tumors in *Drosophila* may be associated with tumor-inducing factors. The *tu-e* tumor-inducing factor has been purified twelve thousandfold by protein-fractionation procedures. These purification procedures removed three modifiers of the tumor-inducing factor. While crude preparations induce benign, free-floating tumors, purified preparations of the tumor-inducing factor induce tumors that are invasive and frequently lethal. These tumors have been successfully tissue-cultured.

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A TUMOR-INDUCING FACTOR IN *DROSOPHILA MELANOGASTER*. II. ITS CHARACTERISTICS AND BIOLOGICAL NATURE*

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With the successful purification of the *tu-c* tumor-inducing factor (TIF),¹ investigations were conducted to determine some of its biological and biochemical characteristics, thus making available some information concerning its role in tumor etiology, the host's susceptibility to it, and the inheritance of TIF.

The characteristics of the *tu-c* donors,²⁻⁴ the *wild 51-52* (genetically tumor free) hosts,^{3, 4} the injection techniques⁵⁻⁶ and apparatus,¹⁻⁵ and the process by which TIF is obtained, purified, and assayed¹ have been described previously. Unless otherwise stated, the preparations of the TIF used in these experiments were highly purified and contained 0.66 μ g. of protein per ml. [2 inductive units (I.U.) per ml.]. Samples were diluted in "Tris" buffer (0.05 *M*, pH 7.8).

BIOLOGICAL STUDIES

Multiplication

One tissue culture and 2 *in vivo* methods were employed to determine whether TIF multiplication was possible.

Tissue culture. Since *Drosophila* tumors have been successfully cultured by a new technique,⁶ a modification of this procedure was employed. This method consisted of the incubation of aliquots of TIF (0.1 I.U. ml.) with trypsinized larval tissue for 24 and 48 hr. After these periods of incubation, the mixtures were centrifuged in a refrigerated centrifuge at 35,000 g for 15 min., and the supernates were assayed. TIF activity increased significantly during incubation in tissue culture. The assay of TIF after a 24-hr. tissue-culture incubation indicated that TIF activity significantly increased (TABLE 1, part 1). TIF activity did not increase significantly during the subsequent 24-hr incubation, however.

In vivo culture. A time study was used in the first *in vivo* method. Approximately 0.015 μ l. of a TIF (0.1 I.U. ml.) solution was injected into each of 900 hosts. These injected hosts were incubated at 25° C. At 2-hr. intervals following the initial injection, crude extracts of 25 injected hosts, diluted 10⁴-fold (10⁻⁴ I.U. ml.) were assayed. After a 12-hr. (*in vivo*) incubation in *wild 51-52* hosts, the activity of TIF had significantly increased (TABLE 1, part 2). Assays of crude extracts of TIF, prepared at 2-hr. intervals, indicated that TIF activity did not increase during the first 2 hr. of

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TABLE 1
ASSAYS OF TIF AFTER TISSUE CULTURE AND *IN VITO* INCUBATION

Incubation (hours)	Hosts	Emerged adults	Tumorous adults	Per cent induction	I.U./ml.
1. Tissue culture					
24	302	115	81	70 ± 4.2	2
48	513	108	78	72 ± 3.5	2
2. <i>In vivo</i> incubation					
2	720	99	27	26 ± 4.3	10^{-4}
4	762	112	48	43 ± 5.1	10^{-2}
6	747	92	48	52 ± 5.3	10^{-2}
8	786	108	60	57 ± 4.7	10^{-2}
10	735	124	72	58 ± 4.4	10^{-2}
12	753	104	62	60 ± 4.8	1

incubation. These 2 hr. of latent TIF multiplication were followed by a 2-hr. period of rapid multiplication (TABLE 1). After this period of rapid multiplication, replication occurred at a significantly slower rate, so that no increase in I.U. ml. was detectable until the end of the twelfth hour of incubation.

The assays of TIF, after tissue culture and *in vivo* incubation, demonstrated that TIF does increase in quantity. The extent of this increase clearly indicated that a high rate of multiplication is a definitive biological characteristic of this TIF. Since multiplication is a known characteristic of virus, a second type of experiment involving serial transmissions concomitant with high dilutions was conducted to demonstrate more conclusively the high rate of TIF multiplication.

Approximately $0.015 \mu\text{l.}$ of a TIF (0.1 I.U. ml.) solution was injected into each of 100 hosts. After a 24-hr. incubation at 25°C. , a crude extract of 25 injected hosts, diluted 10^4 -fold with Tris buffer, was prepared. An aliquot (approximately $0.015 \mu\text{l.}$) of this preparation was injected into each of 100 new hosts. In series A this procedure was repeated for 4 consecutive transmissions. In series B this procedure was repeated for 2 transmissions. The crude extract used in the third transmission was diluted 10^5 , while the next crude extract was diluted 10^4 . By the end of the last transmission and incubation, TIF in crude extracts was at a dilution of 10^{16} (series A) and 10^{20} (series B). The crude extracts of transmissions of series A (dilution 10^{16}), and aliquots of these, diluted 10^3 and 10^7 , were assayed. The crude extracts of transmission series B (dilution 10^{20}) and aliquots of these, diluted 10^3 and 10^6 , were also assayed. In series A the increase in TIF activity, after TIF had been serially transmitted and incubated in 4 groups of hosts, represented a 10^{14} - to 10^{20} -fold increase in I.U. ml. (TABLE 2), whereas the TIF activity in series B represented a 10^{18} - to 10^{23} -fold increase in I.U./ml.

Since there was a 10^4 -fold increase in I.U. ml. in a 12-hr. period of *in vivo* incubation, the 10^{23} -fold increase in I.U. ml., resulting from TIF multiplication in serial transmission (TABLE 2), was not unexpected. The rapid rate

TABLE 2
ASSAYS OF TIF AFTER SERIAL TRANSMISSIONS

I.U./ml. (by dilution)	Hosts	Emerged adults	Tumorous adults	Per cent induction	Assayed I.U./ml.
1. Series A					
10^{-16}	830	96	48	50 ± 5.1	10^{-2}
10^{-19}	920	104	44	42 ± 4.8	10^{-2}
10^{-23}	880	88	31	35 ± 5.1	10^{-3}
2. Series B					
10^{-20}	1225	135	70	53 ± 4.2	10^{-2}
10^{-23}	645	99	42	42 ± 5.0	10^{-2}
10^{-26}	1004	128	44	34 ± 4.2	10^{-3}

(10^4 -fold multiplication in 12 hr.) and the extent (10^{20-23} -fold) to which TIF multiplied may have been stimulated by the physiological reactions in the hosts. A multitude of physiological reactions and developmental changes were occurring in the 5-day postembryonic development of *Drosophila melanogaster*. These reactions and changes were accelerated during the latter part of the third larval instar (96 to 120 hr.). Some of the physiological reactions occurring during this climatic period (prior to ecdysis) are: (1) protein synthesis and histolysis of tissue (protein break-down), with the resultant liberation of amino acids; (2) increased nucleic acid metabolism;⁷ (3) increased titer and activity of the growth and differentiation hormone;⁸ and (4) increased feeding activity of the animal. Thus, the increase in the general larval metabolism, the high level of protein synthesis and amounts of amino acids in the larval hemolymph, the peak nucleic acid metabolism, and the presence of large amounts of nutrients in the larval hosts at the time of TIF incubation may well have collaborated to stimulate the high rate of TIF multiplication.

Virulence

The virulence of TIF increased perceptibly during the serial transmissions. After the second transmission (series A) virulence was indicated (1) by the precocious appearance of induced tumors in most of the hosts (induced tumors usually appear 36 to 48 hr. after the injection of TIF into hosts whereas, in this instance, they were observed in less than 24 hr.), and (2) by the fact that there was a significant decrease in the 24-hr. postoperative host survival from 80 to 90 per cent to 15 to 25 per cent. These virulent characteristics were more pronounced after the third and fourth transmissions. To overcome this tendency, extracts of the second group of hosts were diluted 10^8 - instead of 10^4 -fold. Hosts injected with this diluted extract had a significantly greater survival value (80 to 90 per cent) and did not possess observable tumors. Host survival after the fourth transmission again was significantly lower (30 to 40 per cent), and precocious tumor development had occurred in some of these hosts. These additional data demonstrate that the degree of

virulence may be related to the extent of TIF multiplication. Increased virulence with TIF multiplication is another viral-like characteristic, since there is a correlation between the degree of pathenogenicity and the rate and/or the extent of viral multiplication.^{9, 10}

BIOCHEMICAL STUDIES

Prior to determining the effects of enzymes upon TIF, it was necessary to determine TIF stability to temperature and to pH alterations. The results of these experiments indicated that, up to 35° C., it is quite stable, and that it is most stable between pH 6.5 to 8.0.

Effects of Enzymes upon TIF Activity

The effects of enzyme digestions upon TIF activity were determined by assays of aliquots of TIF (0.66 μ g. of protein ml.-2. I.U.) that had been incubated with the enzymes [trypsin, chymotrypsin, carboxypeptidase, papain, deoxyribonuclease (DNase), ribonuclease (RNase), and lipase] under the appropriate conditions of pH, temperature, time, and or activators.¹¹ Since the substrates of these enzymes were not synthetic, relatively high concentrations of enzymes were used in these experiments. Therefore, it is possible that some of the enzymatic effects may have been due to impurities in these commercial enzyme preparations.

The assay of control aliquots of TIF and control samples of enzyme solutions subjected to the conditions of each enzyme digestion showed that (1) the enzyme preparations had no tumor-inductive activity, and (2) the activity of TIF (2 I.U. ml.) was unaffected by these control conditions. Assays of the incubated TIF-enzyme preparations indicated that TIF activity was significantly decreased by carboxypeptidase and by papain (TABLE 3, part 1). None of the other enzymes had any significant affect upon TIF activity (TABLE 3, part 1).

Since most of the results of preliminary TIF-enzyme experiments were negative, a technique was devised whereby some of the enzymes could affect TIF activity significantly. This technique consisted of incubating TIF, which had been exposed to a subminimal digestion (no significant affect upon TIF activity) by papain, with other enzyme solutions.¹¹ It was determined that a 4-hr. period was optimal for the subminimal digestion of TIF (SD-TIF) by papain (TABLE 4). The incubation of papain-TIF mixtures for 6 hr. caused a twentyfold reduction of I.U. ml., whereas the 1-hr. incubation of SD-TIF (papain-TIF mixtures incubated for 4 hr.) with DNase resulted in a significantly greater reduction of I.U./ml. Thus, the reduction of TIF activity was not due to the effects of the further incubation of TIF with papain. Moreover, the 2-hr. incubation of TIF with papain and SD-TIF preparations with DNase did not significantly affect the percentage of tumor induction, but the 4- and 6-hr. incubations of these mixtures did have a significant effect. Therefore, the enzymes reduced the percentage of tumor induction by affecting the TIF rather than the susceptibility of target tissue.

As controls for the effects of enzymes upon SD-TIF, aliquots of SD-TIF, subjected to the conditions of each enzyme digestion, were assayed. These

TABLE 3
ASSAYS OF TIF PREPARATIONS AFTER INCUBATION WITH ENZYMES

Enzyme	E TIF*	Hosts	Emerged adults	Tumor-ous adults	Per cent induction	Dev/σ from control (71%)	I.U. ml.
1. Effects upon TIF							
Trypsin.....	15/1	909	96	66	69 ± 4.7	1	2
Chymotrypsin.....	15/1	1080	88	64	73 ± 4.7	1	2
Carboxypeptidase..	15/1	1050	93	28	30 ± 4.8	3	10 ⁻⁸
Papain.....	15/1	1162	73	4	6 ± 2.8	3	10 ⁻¹⁰
DNase.....	15/1	1089	135	85	63 ± 4.1	1	2
RNase.....	15/1	930	123	86	70 ± 4.1	1	2
Lipase.....	100/1	773	142	102	72 ± 3.7	1	2
						Dev 'σ from control (64%)	
2. Effects upon SD-TIF†							
Trypsin.....	1/1	821	78	49	63 ± 4.2	1	1
Chymotrypsin.....	1/1	610	144	58	40 ± 4.0	3	10 ⁻² to ⁻⁴
Carboxypeptidase..	1/15	656	97	38	39 ± 5.0	3	10 ⁻² to ⁻⁴
DNase.....	1/1	690	117	42	36 ± 4.5	3	10 ⁻⁶
RNase.....	1/1	582	132	54	41 ± 4.2	3	10 ⁻² to ⁻⁴
Lipase.....	1/1	604	121	70	59 ± 4.5	1	1

* Ratio of enzyme to TIF.

† Subminimally digested TIF.

TABLE 4
ASSAYS OF TIF PREPARATIONS INCUBATED WITH PAPAIN*

Incubation (hours)	Hosts	Emerged adults	Tumorous adults	Per cent induction	Dev/σ from control (71%)
1. Assays of SD-TIF†					
2	410	124	86	69 ± 4.0	1
4	511	106	68	64 ± 4.7	1
6	533	98	52	53 ± 5.0	3
2. Assays of SD-TIF after incubation with DNase (1/1)					
2	786	114	81	71 ± 4.2	1
4	690	117	42	36 ± 4.5	3
6	759	107	36	34 ± 4.6	3

* One part papain/15 parts TIF.

† Subminimally digested TIF.

assays indicated that the activity of SD-TIF (1 I.U. ml.) was unaffected by these conditions. The assays of enzyme-SD-TIF mixtures indicated that DNase, RNase, carboxypeptidase, and chymotrypsin significantly reduced SD-TIF activity, whereas trypsin and lipase had no effect (TABLE 3, part 2).

Sedimentation Studies

Assays of the supernatant fluids and sediments (resuspended with Tris buffer) obtained by the centrifugation of TIF aliquots at 105,000 g for 1 hr. indicated that virtually all of the active TIF remained in the supernatant fluids (TABLE 5, part 1). In contrast, assays of the supernatant fluids and

TABLE 5
ASSAYS OF TIF ALIQUOTS AFTER HIGH-SPEED CENTRIFUGATION

Fraction	Hosts	Emerged adults	Tumorous adults	Per cent induction
(1) After centrifugation at 105,000 g for 1 hr.				
Supernatant fluid.....	700	160	116	73 \pm 3.5
Resuspended sediments.....	1088	224	22	10 \pm 6.4
(2) Centrifugation at 105,000 g for 1 hr. after incubation with lipase*				
Supernatant fluids.....	606	120	15	13 \pm 3.2
Resuspended sediments.....	883	98	67	67 \pm 4.8
(3) Centrifugation of SD-TIF† at 105,000 g for 1 hr. after incubation with lipase‡				
Supernatant fluids.....	370	110	16	15 \pm 3.5
Resuspended sediments.....	369	94	61	65 \pm 4.9

* One hundred parts lipase/1 part TIF.

† Subminimal digestion of TIF by papain.

‡ One part lipase/1 part TIF.

sediments (resuspended), resulting from the centrifugation (105,000 g for 1 hr.) of TIF aliquots that had been incubated with lipase (100 parts lipase/1 part TIF at this ratio, TIF activity is not affected by lipase) for 1 hr. at 30° C. indicated that most of the active TIF had been sedimented (TABLE 5, part 2). Subsequently, assays of sediments (resuspended) produced by the centrifugation of lipase with TIF (100 parts lipase 1 part TIF) at various speeds for various times indicated that all of the active TIF was sedimented at 80,000 g in 20 min. Analyses of these sediments indicated a 2.1- μ g. amino acid-nitrogen content¹² for TIF aliquots (before incubation with lipase and centrifugation), whereas there were 1.9 μ g. of amino acid nitrogen in these aliquots after they had been treated with lipase and centrifuged. This negated the possibility that TIF was sedimented because TIF particles had aggregated with lipase molecules.

Absorption Spectra of TIF

The ultraviolet absorption spectrum of TIF (at pH 7.8) was determined in a Beckman Spectrophotometer.¹¹ The ratio of optical densities of TIF at

280, 260 (177/151) to that of known amounts of protein and nucleic acid¹³ indicated that the nucleic acid content of TIF was less than 5 per cent.

Chemical Composition of TIF

Some information about the chemical composition of TIF may be inferred from the results of the enzyme studies. The proteolytic enzyme papain had a very significant effect upon TIF activity while, in contrast, the proteolytic enzymes chymotrypsin and trypsin had no effect. From these contrasting results and the knowledge that papain affects native animal protein, whereas chymotrypsin and trypsin principally affect denatured protein, it may be inferred that the active principle of TIF contains native protein.

The subminimal digestion by papain may be a slight denaturation of the protein component of the active principle. The denaturation of the protein appears to have made the active principle susceptible to some of the enzymes. The significant effect by chymotrypsin (even at a lower concentration) upon the active principle, after TIF had been subminimally digested by papain, lends credence to the suggestion that (1) protein is a component of the active principle and (2) the protein of the active principle had been denatured by the subminimal digestion by papain. After the protein component of the active principle reached a susceptible state, DNase and RNase (at lower concentrations) significantly reduced TIF activity. These results infer that DNA and RNA are components of the active principle of TIF. Analysis of the ultraviolet absorption spectrum of TIF demonstrated the presence of up to 5 per cent of nucleic acid.

Since a high concentration of lipase did not significantly affect TIF activity, it may be concluded that lipid is not a necessary component of the active principle. Nevertheless, the results of the sedimentation studies, in which the incubation of TIF with lipase was critical, possibly indicates that lipid may be a component of TIF.

TIF appears to contain protein, nucleic acid, and lipid. These components have been repeatedly demonstrated in animal viruses. From this similarity, the fact that TIF has virulent and pathogenic (induction of invasive and often lethal tumors⁶) characteristics, and the extent to which TIF multiplies, it may be concluded that the TIF extracted from the *tu-c* strain of *Drosophila melanogaster* is a tumor-inducing virus.

INHERITANCE OF TIF

The Tu-e Strain

The inheritance of the pigmented tumor in the *tu-c* strain has been associated with a "recessive gene" on the right arm of the second chromosome.² The role of this "tumor gene" in tumor formation is not known.

Animals of the *tu-c* strain were reciprocally outcrossed (virgin matings) to animals of two genetically tumor-free strains (*Canton-S* and *wild 51-52*). After examining and dissecting more than 8000 hybrids from these crosses, it was determined that, although the F₁ hybrids were heterozygous for the recessive tumor gene, 4 per cent of these animals were tumorous. Moreover,

assays of crude extracts of F_1 hybrid larvae indicated that these extracts contained an active tumor-inducing factor. Furthermore, there was no significant difference in tumor incidence or extractable TIF of hybrids, regardless of the sex of the *tu-c* parent (unpublished data). These data indicate that TIF may be inherited extrachromosomally.

Induced-Tumor Host Strains

Although the *wild 51-52* strain is genetically stable, tumor-free, and does not contain any active tumor-inducing factor, three induced-tumor strains were derived from it (unpublished data). Tumorous and nontumorous parents were selected from *wild 51-52* hosts that had been injected with purified TIF. Strain A was derived from hosts with induced tumors, whereas strain B was derived from tumor-free injected hosts. Strain C was developed by mass selective matings of tumorous animals from the F_3 progeny of strain B and was maintained by selective matings of tumorous progeny.

Although there was no tumor incidence in F_1 progeny of strains A and B, there was some active TIF in the F_1 larval extracts (FIGURE 1). More active TIF was present in F_2 larval extracts, and there was a 2 per cent tumor incidence in the F_2 generations of both strains. Tumor incidence was greater in the F_3 progeny in strain A and was significantly higher in strain B. Moreover, more TIF was present in the larval extracts of strain-B progeny. It may be noted that, after selective matings of tumorous F_2 adults of strain B, the incidence was approximately threefold greater than in the progeny of the unselected matings of strain B. Thereafter, tumor incidence in both strains A and B remained fairly constant, even though the amount of extractable TIF varied from generation to generation (FIGURE 1).

The tumor incidence in the progeny (F_1 , strain C) resulting from the mass selective mating of F_3 tumorous progeny (strain B) was almost 100 per cent, and the active TIF content in strain C extracts was comparatively high. Thereafter, although the amount of TIF varied in strain-C progeny, the incidence of tumors was constant.

It appeared that the amount of TIF had increased in strains A and B. It may be concluded, however, that the degree of tumor incidence in these strains was not wholly dependent upon TIF concentration. This indicates that susceptibility to TIF in these strains may play an important determinative role in tumor formation. Susceptibility may be governed by gene complexes. This hypothesis appears to be supported by the tumor incidence in the F_1 progeny (strain C) of selected tumorous parents. Thus, this selection for tumors may have reflected an unintentional selection for genes that increased the susceptibility of animals to the tumor-inducing factor. However, matings of tumorous parents of the F_3 generation of strain A did not alter tumor incidence in the next generation (FIGURE 1). This indicates that selection for tumors is *not* concomitant with selection for genes that increase susceptibility.

The differences in susceptibility among these strains may be the result of varying amounts of inhibitory modifiers of TIF activity, since such modifiers have been demonstrated in the *tu-c* tumor strain.¹ The presence of these

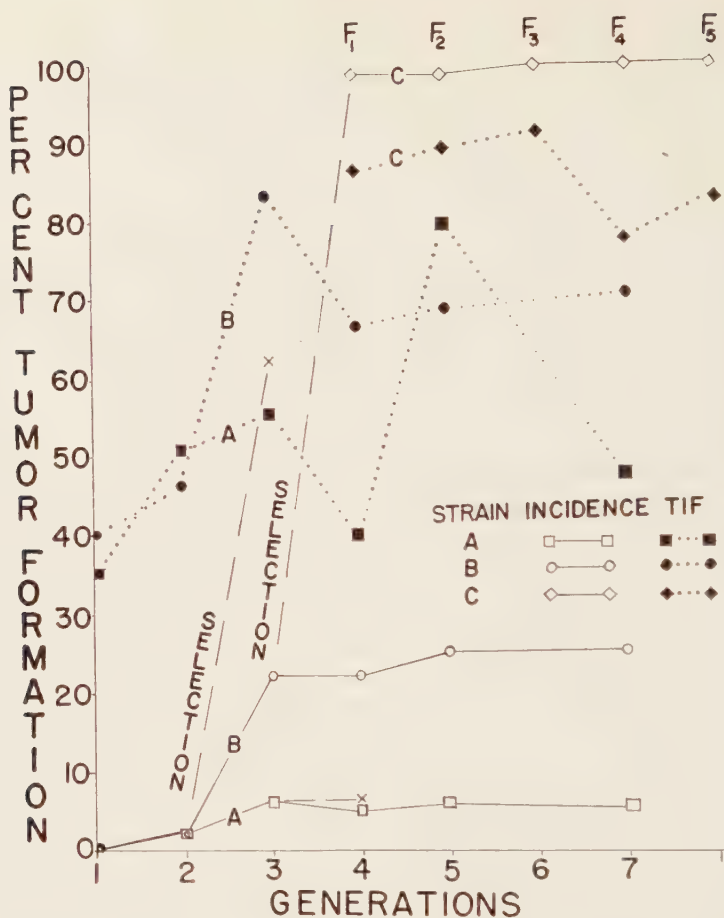


FIGURE 1. Effects and vertical transmission of the *tu-e* TIF in induced-tumor (*wild 51-52*) strains.

modifiers in these strains and the possibility that they are gene-determined is yet to be demonstrated.

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CYTOPLASMIC INHERITANCE*

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Growth and Differentiation

Cytoplasmic inheritance is exemplified in the differentiation of tissue in Metazoa and Metaphyta. We all know what is meant by cytoplasmic inheritance; although this expression is useful, the term "extrachromosomal" heredity may be preferable. Growth (involving cell division) and differentiation occur concurrently and are obviously interdependent. Animal cells do not have a cell wall; therefore, in differentiation (ontogeny) the cytoplasm is halved at each division. When division occurs without concurrent growth, as in the cleavage of marine eggs, each cell becomes smaller and the cytoplasm is apportioned equivalently to each daughter cell. In this process the karyoplasmic ratio is maintained and both cells and nuclei become smaller as the division proceeds.

The Diphasic Nature of Growth

Boveri¹ has stated: "The constant, which we must accept as something given and not at present further analyzable, is the fixed proportion between nuclear volume and protoplasmic volume, namely, the karyoplasmic ratio." According to Wilson² (p. 730), "Boveri's extensive measurements led him to the unexpected result that in the sea-urchin, with increasing chromosome-number the nuclear volume increases more rapidly than the cytoplasmic, in such a manner that it is the surface of the nucleus and not its volume that is directly proportional to the number of chromosomes." Wilson has stated further (p. 272): "In dwarf larvae of different size, arising from isolated blastomeres of the 2- or 4-cell stages, the cells are of nearly or quite the same size but differ in number, the $\frac{1}{2}$ -larvae containing about one-half, and the $\frac{1}{4}$ -larvae about one-fourth the normal number characteristic of an entire egg at the corresponding stages. Conversely, in giant larvae produced from two fused eggs, the number of cells is double the normal number. In all these cases the result is brought about by an adjustment of the cleavage process to the size of the embryo; the smaller the original piece the fewer the cleavages required to produce cells and nuclei of the proper size. Morgan and Driesch thus reached the conclusion that the cleavage is so regulated as to produce a fixed or typical cell size at a given state rather than a fixed number of cells; and this was afterwards confirmed by Boveri.

"In the foregoing cases the volume of the nucleus varies primarily with that of the cytosome or cytoplasmic mass. Equally interesting is the reverse case in which the primary variable factor is the nuclear mass. Typical cases are offered by the tetraploid giant forms of *Spirogyra*, *Oenothera*, *Primula* and *Solanum*, already mentioned in which the nuclear volume is doubled as a

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result of a doubling of the number of chromosomes, and the normal karyoplasmic ratio is restored by a corresponding growth of the cytosome to twice its former size. The classical experiments on this subject are those of Boveri on sea-urchin eggs in which the number of chromosomes may readily be altered experimentally in several ways . . . " Wilson (p. 732) continues: "These various facts, together with those earlier considered show that although the karyoplasmic ratio is a real and important cell constant it results from a complex of factors often difficult to analyze. It calls, therefore, for critical treatment in all attempts to employ it as a factor in age, cell division or the like or as a guide to the number of chromosomes that have entered into the composition of the nucleus.

"Finally, the remarkable fact may be emphasized that not only the nuclear size but also that of the formed components of both nucleus and *cytosome* often varies proportionately to that of the cytoplasmic mass. This is seen in the mitotic figures of dividing blastomeres and the centrosomes or "centrospheres" of the interphase; in the centrioles, chondriosomes, acroblast and chromatoid bodies; and in the plastids. These differences evidently result from the increased size of the cytosome, however caused; for they appear in the polymegalous spermatocytes of insects, which are diploid as well as in the tetraploid cells of *gigas* forms. The fact may also here be recalled that the size of the plasmosomes varies with that of the cytosome and nucleus, and that the same is true in certain cases of the chromosomes (during cleavage), though this seems not to be invariable."

Cytoplasmic Growth at a Linear Rate

The important fact established by the early cytologists is that growth is diphasic. Nuclear growth and cytoplasmic growth are competitive and mutually exclusive. The fact that both cytoplasmic and nuclear organelles vary in size with the total volume of the cytoplasm complicates the determination of cause and effect. An important difference between plant cells (such as yeast cells) and animal cells is that the cell wall establishes the original size of the cell initiating a clone and, when division occurs under conditions in which growth is restricted, it is not possible to reduce the size of the original cell.

When yeast cells grow in an adequate liquid medium with full aeration and agitation under conditions in which the nutrients are constantly maintained at full strength, the sizes that the cells attain at the ends of their growth periods are generally quite uniform. On the other hand, when yeast cells are grown in a colony on an agar surface, even though the medium is quite adequate, there are enormous variations in the sizes of the cells.³ This condition arises on a solid medium because of inequalities in the accessibility of nutrient and oxygen to the cells; as budding progresses, the starved cells become smaller and smaller.

Kennedy⁴ and Lindegren and Haddad⁵ found that the increase in volume of the individual yeast cell begins abruptly with the appearance of a bud and continues uniformly in a linear manner, terminating abruptly when final volume is achieved. During this period the volume of the mother cell remains

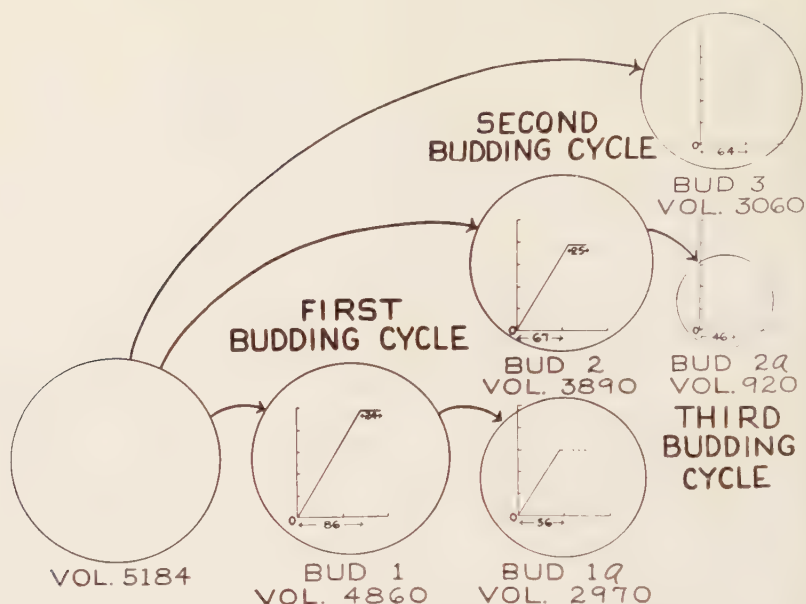


FIGURE 1. Graphic representation of the budding cycles and the increases in volumes of cells under conditions of limited nutrition. The cells are represented by circles; the areas are proportional to the volumes of the cells. The original cell (at the left) produces bud 1 in the first budding cycle, bud 2 in the second budding cycle, and bud 3 in the third budding cycle. Bud 1 produced bud 1a in the second budding cycle, and bud 2 produced bud 2a in the third.

The graphs inscribed in each circle represent the growth rates (volume on the Y axis, time on the X axis). The original cell produced cytoplasm for bud 1 for 86 min. and ceased to increase in volume. During a 24-min. lag period a new nucleus was synthesized and, thereupon, bud 1 began to synthesize cytoplasm producing bud 1a. Bud 1 was able to sustain cytoplasmic synthesis for the production of bud 1a for only 56 min., and bud 1a failed to bud during the next 90 min., suggesting that it had obtained insufficient materials from bud 1 to complete the synthesis of a new nucleus, thus precluding the possibility of another cytoplasmic cycle.

During the second budding cycle the original cell was able to sustain cytoplasmic synthesis for only 67 min., producing bud 2. Bud 2 remained at constant volume for 25 min., during which a new nucleus was synthesized. In the third budding cycle, bud 2 synthesized cytoplasm for 46 min., at a very low rate, producing bud 2a, which failed to bud during the next 90 min. During the third budding cycle the original cell produced bud 3 by synthesizing cytoplasm for 64 min., but bud 3 was unable to produce another bud, presumably because it did not obtain sufficient material to synthesize a new nucleus.

In every instance the growth rates were linear and, except for bud 2a, which grew at an extremely low rate, were approximately the same. The size of each bud produced by the original cell diminished progressively during each budding cycle, and every bud was smaller than its mother cell.

constant. In this study, growth was followed through three budding cycles. The graph within each circle (FIGURE 1) shows the rate of growth during the growth period and the period of rest before the next bud is produced. It appears that the contents of the mother cell are not partitioned equally between mother and bud, even when the daughter cells are approximately equal in volume to those of the mother cell. This is inferred from the fact

that, although bud 2 produced a bud in the third budding cycle, bud 1a did not. Lindgren and Haddad⁶ developed a method for determining the density of yeast cells. They examined the density of these cells during the period of constant volume before budding,⁷ found fluctuations between 1.05 and 1.08 in density, and inferred that little, if any, increase in total weight could have occurred.

Ogur, Minckler, and McClary⁸ analyzed yeast cells for their deoxyribonucleic acid (DNA) content during the budding cycle. If each cell with a bud were counted as two cells (even when the bud was so small that it was only barely perceptible), the DNA content per cell was a constant, showing that at the first appearance of the bud the DNA has already doubled and, therefore, that the synthesis of all the DNA necessary for the formation of the new nucleus had occurred. Sarachek⁹ recorded the killing effect of ultraviolet radiation on yeast cells undergoing different phases of the budding cycle. The cells were irradiated at various stages during the initial period of synchronous division. At the time of the appearance of the first bud, the cells contained twice as many radiosensitive targets as the original unbudded cells. These data indicate that, if the radiosensitive sites were in the nucleus, a complete new nuclear apparatus was synthesized during the period of constant volume, presumably after cytoplasmic growth had ceased.

It is inferred that the growth of yeast cells is diphasic. During the increase in volume only the cytoplasmic, or extrachromosomal, material is synthesized, while during the period of constant volume only nuclear material is synthesized. Cytoplasmic synthesis occurs in a linear manner with time until final volume is attained. Since the rate of increase during cytoplasmic growth is constant, the concentration of enzymes controlling growth likewise remains constant during the cytoplasmic increase.

Bud 1 grew for 86 min. and, after a 24-min. rest period (for the synthesis of its nucleus), produced bud 1a. Bud 1a ceased to grow after 56 min., but failed to produce a second bud during the next 90 min. Bud 2 produced cytoplasm for 67 min. and required a 25-min. rest period for the synthesis of its nucleus. Bud 2a ceased to grow after 35 min., indicating depletion of nutritional materials. Since ribonucleic acid (RNA) is known to be primarily a cytoplasmic component, while DNA is supposed to be almost exclusively a nuclear component, recent advances in knowledge of the molecular components of DNA and RNA may make it possible to speculate concerning the molecular basis for the diphasic nature of growth. These two substrates differ in that RNA contains ribose and uracil, while DNA contains deoxyribose and thymine. The mutually exclusive synthesis of these two fundamental materials could be achieved by any mechanism that prevents the synthesis of uracil and ribose and initiates the synthesis of thymine and deoxyribose, or vice versa. Seymour Cohen¹⁰ has provided a molecular model for part of this process.

Differentiation

It seems clear that differentiation must occur during the cytoplasmic phase of growth. I propose to distinguish between normal and pathological mechanisms of differentiation (cytoplasmic inheritance). The orderly, directed

(nonpathological) differentiation of tissue appears to be initiated and achieved without detectable change in the genome, and to be perpetuated for an indefinite period. Tissues that have been differentiated in the body may dedifferentiate *in vivo* to produce cancers or other abnormalities, or may dedifferentiate *in vitro* (in tissue culture).

Warburg¹¹ has proposed a hypothesis of differentiation that includes both the normal, orderly type and the pathological process that leads to the development of cancer. He proposed that the materials that maintain the integrity of differentiated tissue are produced by the localized use of oxidative energy at specific sites on cytoplasmic granules. Loss or degradation of the granules could result in dedifferentiation, provided the cell became adapted to anaerobiosis and by this process could obtain the energy necessary for survival. Contamination of the surface of the granules by homologues of oxidizable materials could lead to the degradation of the oxidative apparatus and a consequent loss of tissue specificity. Lindegren and Hino¹² made a study to determine if inactivation of the aerobic mechanism by anaerobiosis could bring the latent anaerobic mechanism into full expression and lead to irreversible degradation of the aerobic mechanism, and thus to dedifferentiation.

Culture 8256 of the Carbondale breeding stock is a respiratory-sufficient (AER), adenine-deficient, pink culture. It produces respiratory-deficient (aer) colonies that are brownish-orange,¹³ in contrast to the bright pink of the AER colonies. This change from pink to brown is irreversible, but all pink clones always produce both pink and brown subclones.

An experiment was performed to determine if exposure to anaerobic conditions *in the absence of growth* would suffice to increase the frequency of aer cells in a population. Aggregation of the cells in this experiment would not be important, since no difference in dispersal of cell aggregates would be expected under either condition. No significant difference in the frequency of aer cells was observed, either in aerobiosis or anaerobiosis, and it was inferred that, in the absence of growth, anaerobiosis does not bring about loss of respiratory ability. A similar result was obtained when sugar alone was added to the buffer, showing that, in the absence of division, this type of change does not occur in an actively fermenting cell.

The Production of aer Cells During Growth Under Anaerobiosis

Determination of the rate of loss of respiratory ability (AER to aer) would not be feasible if the aer cells divided more rapidly than the rate at which they originated from AER cells. When standard nutrient medium is made up without carbohydrate, aer cells multiply either very slowly or not at all.¹⁴ An experiment was performed using nutrient broth (without added carbohydrate) in place of buffer. The colony counts in TABLE 1 show a significant increase in the percentage of aer cells under anaerobiosis as compared with aerobiosis. The final row of figures in TABLE 1 shows that, under similar conditions, aer cells grow very poorly, if at all.

One of the complications in this analysis arises from the fact that haploid cells tend to aggregate. If the colonies of aer cells arose primarily from single

cells, while those of AER cells arose from aggregates, a misleading conclusion might be drawn. TABLE 2 reveals the state of aggregation of the cells in the experiment described in TABLE 1. Although aggregation decreased as growth progressed, the rates were so similar under aerobiosis and anaerobiosis that the effects could be inferred to have canceled out. From these data it is possible to correct the observed frequencies of aer cells as indicated in the last row in TABLE 2. Anaerobiosis produced a tenfold increase in the population of aer cells; moreover, since the latter do not grow in this particular medium, the increase must have resulted from a change of AER cells to aer cells.

TABLE 1
COUNTS OF RESPIRATORY-DEFICIENT AND RESPIRATORY-SUFFICIENT CELLS
AFTER AEROBIC AND ANAEROBIC GROWTH IN NUTRIENT MEDIUM
WITHOUT ADDED CARBOHYDRATE

Time (days)	Aerobiosis			Anaerobiosis	
	0	1	2	1	2
A. Original culture: 8256 AER					
Cells per ml. of culture solution					
Total	2.0×10^7	1.2×10^6	1.9×10^6	4.3×10^5	1.0×10^6
aer.....	3.4×10^2	6.0×10^3	1.3×10^4	1.6×10^4	4.8×10^4
Percentage aer.....	1.6	0.5	0.6	3.7	4.8
B. Original culture: 8256 aer					
Cells per ml. of culture solution	2.4×10^3	—	3.7×10^3	—	2.7×10^3

TABLE 2
HEMOCYTOMETER COUNTS DURING AEROBIOSIS AND ANAEROBIOSIS

Time (days)	Aerobiosis		Anaerobiosis	
	1	2	1	2
Total aggregates counted.....	34	91	53	64
Single cell.....	13	64	15	38
Cluster.....	21	27	38	26
Per cent single cell.....	38.2	70.3	28.3	59.3
No. of cells in total aggregates.....	≈400	≈350	≈400	≈200
Corrected percentage of aer cells.....	0.50	0.30	5.00	3.80

An Improved Method for Testing for Respiratory Deficiency

Ogur and St. John,¹⁵ following a suggestion by Susumu Nagai* based on work done in Osaka University, Osaka, Japan, by S. Nagai, N. Yanagishima, J. Hiraoka, and H. Takada under the direction of J. Ashida, improved upon the diagnosis of aer cells in AER populations by the following procedure:

About 100 cells are spread on the surface of a peptone, liquid-yeast-extract agar plate containing 1 per cent glucose. After the colonies have developed they are overlaid with about 20 ml. of 1.5-per cent agar containing 1 mg. per ml. of 2,3,5-triphenyltetrazolium chloride. Colonies with an intact aerobic mechanism turn a deep red within 1 to 3 hr., while those incapable of oxidizing carbohydrate retain their original color or turn a very light pink. A control on the preceding experiment confirmed the diagnosis of pink colonies as AER and the light-brown colonies as aer.

Ephrussi¹⁶ found that, by using acriflavine, it was possible to destroy the oxidative apparatus in 100 per cent of the yeast cells treated, without otherwise impairing their metabolic apparatus, and that destruction of the respiratory apparatus was irreversible. It has since become evident that, in addition to acriflavine, a great variety of materials will increase the rate at which this defect occurs in yeast cells.

Other Experiments on Respiratory-Deficient Yeasts

Ephrussi described gene-controlled inheritance of the respiratory apparatus based on the observation of 2:2 tetrad segregations for respiratory-deficient to respiratory-sufficient. An extensive experience with regular and irregular segregations in *Saccharomyces* has demonstrated that authentication of gene-controlled heredity requires the analysis of hybrids marked for a variety of other characteristics. Our own experience has revealed few instances of gene-controlled respiratory defects in *Saccharomyces*.

Ogur *et al.*¹⁷ analyzed a number of pedigrees of the "heterozygote" AER \times aer and found the overwhelming predominance of either AER AER AER AER or aer aer aer aer tetrads. This finding was subsequently confirmed on many occasions by G. Lindegren, M. Ogur, and R. St. John. The "heterozygous" mating tends to produce more aer aer aer aer than AER AER AER AER tetrads, suggesting that grana lacking cytochrome oxidase are either incompatible with or usually outgrow those containing it. AER \times AER hybrids occasionally produce aer tetrads. This is consistent with our procedure of mass matings, since an occasional aer variant may be expected to arise spontaneously. Matings of aer \times aer do not produce spores.

Genic Control of Differentiation

Since O. Warburg assumed that tissue integrity was controlled only by an extrachromosomal mechanism, confirmation of his view that anaerobiosis leads to degeneration of the aerobic mechanism does not exclude the possibility that cancer may arise from other causes. The possibility that gene mutation may effect dedifferentiation has been raised by Beerman's¹⁸ study of the

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polytene chromosomes of *Chironomus*. Beerman found that the "puffs" in polytene chromosomes originate at single bands. It is generally accepted that the puffs indicate that the gene is producing its specific gene product. Beerman showed that cells from different tissues exhibit characteristic patterns of "puffing" specific for the tissue. This suggests that different combinations of genes control tissue specificity, and it follows that inactivation of any one of the specific genes controlling differentiation of a specific tissue would lead to dedifferentiation of the cell. This view is consistent with Yosida's¹⁹ findings of V-shaped chromosomes in the tumor cells in rats and mice, since the fusion of nonhomologous chromosomes might produce position effects disturbing gene function.

Adaptive Enzyme Formation

The phenomenon of adaptive enzyme formation in yeast has shown that the adaptation to the fermentation of several sugars, especially galactose, melibiose, and maltose, requires a preliminary period of exposure to the substrate. In addition to exposure to the substrate, an external or internal source of energy is necessary to achieve the synthesis of the adaptive enzyme. Dissimilated yeast cells exposed to pure galactose (freed of alcohol and glucose, both of which are common contaminants of C.P. galactose) do not develop an adaptive enzyme. The addition of a small amount of glucose initiates the adaptive process, and a dissimilated yeast exposed to galactose for about three hours achieves full fermentative ability without cell division. Different adaptations require different adjuvants. Cultures that ferment maltose adaptively require both glucose and a source of nitrogen, in addition to maltose, to produce enzyme. Unadapted yeast cells contain no trace of the adaptive enzyme; it is only the presence of the inducer and an adequate nutritional background that makes it possible for the cell to produce this substance.

Experiments on the Cytoplasmic Transmission of Adaptive-Enzyme Formation

Several years ago Spiegelman *et al.*²⁰ suggested that, provided that the adaptive enzyme and its substrate were present throughout the life cycle, the effects of genic adaptation could be transmitted through several cell generations in cells lacking the gene controlling the synthesis of the enzyme. This suggestion was an attempt to explain aberrant segregational ratios. Three experiments were described:

(1) A diploid hybrid heterozygous for the ability to ferment melibiose (ME me) was made. The first exposure of the haplophase segregants to melibiose occurred after the cultures were fully grown. All asci segregated regularly (ME ME me me).

(2) A similar hybrid was produced and brought to sporulation in constant contact with melibiose. The asci were all ME tetrads.

(3) When the haplophase cultures from the second experiment were dissimilated for from seven to twenty days in phosphate buffer in the absence of melibiose, two from each ascus lost the ability to ferment melibiose.

On the basis of these data we concluded that some cells acquired the ability

to ferment melibiose through cytoplasmic transfer of the enzyme from the dominant form. Several subsequent experiments on the effect of continued exposure to substrate yielded negative results; there was no significant increase in the number of asci containing four fermenting spores in the presence of the substrate over the number of similar asci obtained from heterozygotes developed in the absence of the substrate. The results were interpreted on the assumptions (1) that the genes were stable throughout the life cycle and that a heterozygous diploid should produce only a 2:2 gametic ratio; (2) that only diploid zygotes were produced; (3) that the substrate does not induce mutation to the dominant gene; and (4) that dissimilation does not affect the expression of the gene. Other interpretations were made possible by the demonstrations that genes in the heterozygous condition may undergo conversion; that hybrids between haploids often contain a few tetraploid zygotes; and that the specific substrate may induce gene mutation to the dominant allele. At the time it was performed, the crucial aspect of the experiment was the fact that dissimilation of the fermenter cultures in buffer in the absence of melibiose led to the loss of ability to ferment melibiose. A critical investigation of this phenomenon revealed the fact that the ability to maintain a gene in its *functional* capacity depends upon adequate nutrition. To express this in a more conventional manner reverse mutation from the functional to the nonfunctional aspect of the gene occurs with high frequency in conditions of poor nutrition. These experiments are reported by Lindgren²¹ (TABLE 26-9).

Not only can gene-controlled abilities for the splitting of a disaccharide be lost by dissimilation, but Lindgren and Skoog²² showed that yeasts incapable of fermenting glucose can be produced by growth on lactate and synthetic medium, although these variants revert to glucose fermentation rather readily. These experiments indicate that the assumption (No. 4 above) that dissimilation does not affect the expression of the gene is not correct.

Cytoplasmic Transfer During Copulation

It was shown by Fowell²³ that supposed hybrids often produce haploid buds without undergoing sporulation. This observation makes it possible to study the effect of cytoplasmic transfer in yeasts. Zygotes of *Saccharomyces* begin their life cycles as dikaryons instead of true hybrids with diploid nuclei (FIGURE 2a). Plasmogamy is followed by nuclear division, and karyogamy is delayed (FIGURE 2b). The two daughter nuclei pass into a bud produced at the point of union of the two gametes, and the daughter nuclei fuse in the bud to produce a diploid nucleus (FIGURE 2c); the original dikaryotic zygote continues as a dikaryon and produces a second bud (FIGURE 2d). This bud also receives a pair of nonsister nuclei produced by the mitotic division of the two nuclei of the original dikaryon. Simultaneously, the diploid nucleus produces a diploid bud. Since the original dikaryon persists indefinitely, it may occasionally produce hybrid buds identical to the original gametes that presumably contain a mixture of cytoplasm from both parents.

The somatic separation of a dikaryon into its parental components without a reduction division has been encountered frequently in this laboratory, espe-

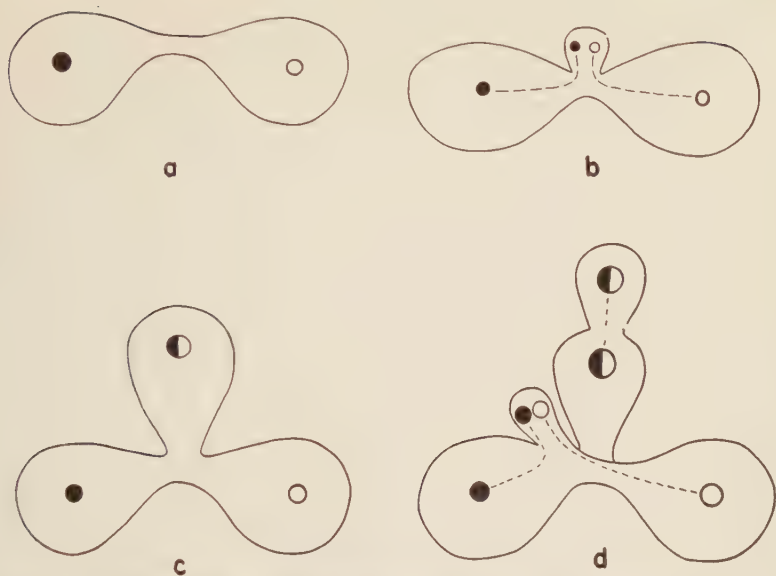


FIGURE 2. Diagrammatic representation of the steps in the production of a hybrid from fused yeast gametes. The nuclei are represented schematically as circles. In *b* the daughters of the original gametic nuclei have migrated into the bud. In *c* the nuclei in the hybrid bud have fused. In *d* the fused hybrid nucleus has divided, the original gametic nuclei have divided again, and their daughter nuclei have migrated into the bud.

cially in attempts to produce stable triploids. This phenomenon must be taken into account in analyzing the data from cases of supposed somatic segregations. It was exploited to study the possibility of transferring a cytoplasmic enzyme into a cell that did not contain the gene controlling the synthesis of the enzyme. A diploid cell homozygous for mating type and carrying at least one GA gene capable of inducing the enzyme controlling the synthesis of galactozymase was mated to a recessive haploid under conditions in which some galactose was always maintained in the medium. The diploid or dikaryon was cultured in nutrient medium containing galactose, and the haploid buds were removed and transferred to galactose broth. None of these haploid cultures (which should have contained the cytoplasm from the adapted diploid) were capable of fermenting galactose. This negative experiment failed to support the view that an adaptive enzyme could be maintained in the absence of the gene controlling its synthesis. A difficulty in using this technique for studying cytoplasmic transfer is emphasized by the analysis of pedigrees "heterozygous" for AER and aer, which suggests an "all-or-none" type of transmission and may be consistent with the adhesion to the nucleus of the specific extrachromosomal organelle or the competitive elimination of one or the other during growth. Such phenomena might prevent an eventual, uniform mixture of cytoplasmic components; it must be recognized that the fundamental assumption in the interpretation of cytoplasmic transfer is that a uniform mixture has been made.

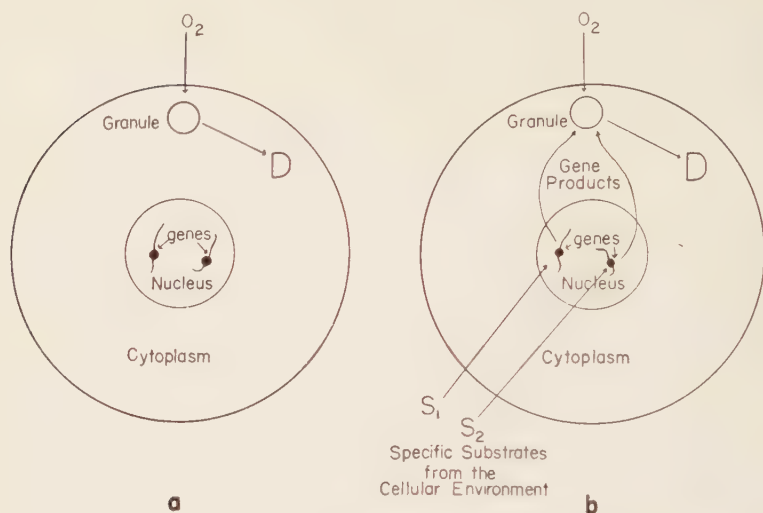


FIGURE 3. (a) Warburg's theory of tissue differentiation. Tissue differentiation is achieved by an oxidative reaction on the cytoplasmic granule, resulting in D, which differentiates the specific tissue. (b) Modified theory of tissue differentiation. Adaptive gene products produced in a specific cellular environment are oxidized on the surface of the cytoplasmic granule to produce D. The destruction of either the specific genes or the granule could produce a "sleeping" cancer cell.

A Modified Theory of Differentiation

On the basis of Beerman's work¹⁸ and what is known of adaptive enzyme formation, Warburg's¹¹ concept may be modified as follows: tissue differentiation may be achieved by the adaptive response of genes to a specific cellular environment (FIGURE 3). "Ontogeny may involve a series of (gene-controlled) adaptive reactions each of which results in a shift in equilibrium, conditioned by the changed environment which continued multiplication produces."²¹ The materials that maintain tissue specificity may be elaborated in the cytoplasm (only by oxidative metabolism) at specific sites on the cytoplasmic granule (as Warburg supposed), but the oxidizable material may arise through the action of gene products that are produced when the cells are placed, through growth and development, in a specific adaptive environment. Dedifferentiation may occur either (1) as Warburg proposes, by degradation of the cytoplasmic aerobic mechanism, or (2) by destruction of the genes that produce the specific materials, the oxidation of which controls the maintenance of tissue specificity. This hypothesis is consistent with the genetic control of cancer and the occurrence of cancer cells with an intact oxidative mechanism.

Other Examples of "Cytoplasmic" Inheritance

Many attempts have been made to interpret the phenomenon of differentiation in terms of "cytoplasmic" inheritance. Most of these seem to

involve virus infection rather than an extrachromosomal component integrated into the cell. This view recognizes the fact that the increased complexity of the extrachromosomal apparatus has been achieved by the integration into a single system of originally extraneous structures that have become essential to the survival of the organism.

Darlington²⁴ chose as a prime example of a "plasmagene" the paracrinkle virus, which is without symptom in the King Edward potato, but can be transmitted by graft to Arran Victory. The distinguishing characteristic par excellence of plant viruses is the fact that they are not transmitted through the egg. When Carson *et al.*²⁵ made hybrids of King Edward (♀) by Flour Ball (♂), they found that, although the hybrid was capable of infecting scions grafted to it, the seedlings were not infected with the virus. Thus, the paracrinkle virus is clearly not a plasmagene, but a true virus (having a characteristic common to plant viruses, namely, the inability to pass through the egg), despite the fact that it can be transmitted only by graft. It is obviously not a plasmagene, since plasmagenes are *a priori* inherited maternally.

Many plant viruses have nearly reached a symbiotic relationship with their hosts, or do them so little harm that they persist with little effect on the host's survival. Minchin²⁶ first proposed the view that the chloroplasts are evolved from independent green algae that have become so completely adapted to the cytoplasm of green plants that they are incapable of independent existence and have, in turn, made their hosts completely dependent upon them. Woods and duBuy²⁷ have presented abundant evidence indicating that the viruses of green plants are derived from chloroplasts; their arguments are based on the wide distribution of viruses and their spread through the plant by cell division rather than by invasion. On the other hand, the paracrinkle virus that Darlington chose as an example has clearly been identified as a true virus, and his whole argument was based on the contrary opinion.

The kappa phenomenon in paramecium seems more easily understood if interpreted as the survival of the naked genome of a parasite in a foreign cytoplasm rather than as an example of "cytoplasmic inheritance." Only the fact that this parasite affected the cytoplasm instead of the nucleus has led to consideration of the kappa phenomenon as an example of cytoplasmic inheritance. Kappa may be the chromosomal apparatus of a parasite that has lost its own cytoplasm, rather than an integrated cytoplasmic component of paramecium. On this view, kappa is an extraneous structure which confers survival value on paramecium by the development of immunity to its own poisons—just as infection with cowpox renders a human immune to smallpox. The undoubted value of cowpox to the human does not make the virus an integral part of the human. The "imperfect" integration of kappa into paramecium suggests that, since its only value lies in its ability to protect against itself, it may never become a permanent component of the genus.

The sensitivity of *Drosophila* to CO₂, discovered by l'Heritier,²⁸ also provides a demonstration of virus infection by an extraneous agent rather than an example of mechanism involved in the transmission of extrachromosomal characteristics.

Summary

Tissue differentiation exemplifies cytoplasmic inheritance and, since growth and differentiation are interdependent, the diphasic nature of cellular growth is an important aspect of differentiation. Nuclear growth and cytoplasmic growth are mutually exclusive, and cytoplasmic growth occurs at a linear rate, indicating that the enzymes necessary for cytoplasmic synthesis are maintained at a constant concentration. One of the devices for controlling the shift from cytoplasmic to nuclear synthesis may be a shift from RNA to DNA synthesis. Warburg's¹¹ theory of tissue differentiation suggests that the materials that maintain the integrity of differentiated tissue are synthesized oxidatively on cytoplasmic granules. Loss of respiratory ability by the granules would lead to dedifferentiation and possibly to cancer. Warburg's suggestion that anaerobiosis might lead to loss of aerobic ability has been confirmed experimentally. Beerman¹² found that different genes are active in different tissues, suggesting that the environment in which the undifferentiated tissue cell finds itself may lead to adaptive enzyme formation, and that the adaptive enzymes may function in tissue differentiation. Experiments investigating the possibility that cytoplasmic transfer of an adaptive enzyme might maintain the enzyme in the absence of the gene were negative, suggesting that the gene is essential for adaptive enzyme formation and, consequently, for tissue differentiation. The paracrinkle virus, the kappa phenomenon in paramecium, and the sensitivity of *Drosophila* to CO₂ are considered to be examples of virus diseases rather than examples of cytoplasmic inheritance. The loss of oxidative ability in yeast is not of direct significance in the phenomenon of tissue differentiation.

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TRANSPLANTATION OF SUBCELLULAR PARTICLES BY MICRURGY*

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Aims and Limitations

Micrurgical procedures permit the direct visualization of the part of a cell that is transplanted as well as the position inside the host cell into which the structures are transplanted. The action of the particles on the host cell, or vice versa, can be directly studied. With these procedures there can be no question that one is transplanting subcellular particles or dealing with something less than an entire cell.

Several factors prevent an extensive application of subcellular transplantations. The most serious of these are as follows: (1) the procedures must be performed at high-power microscopic levels; (2) the cells must be sufficiently large to allow micrurgical procedures, thus excluding bacterial cells; (3) the cells must be amenable to micrurgy, that is, their extraneous coats must permit penetration by sharp micropipettes; (4) the cells must possess some singular attribute that is recognizable not only in the subcellular particles but also in their action on the host cell; (5) if the effects on either the transplanted particles or the host cells are delayed, then the procedures are limited to those cells that can be kept alive for periods ranging from several hours to several weeks; and (6) the instrumentation is complex and expensive and the procedures are difficult.

Examples of Subcellular Transplantation

Removal and transposition of the micronucleus in Euplotes. Taylor and Farber¹ were the first investigators to remove a subcellular structure from a cell and replace it successfully. The micronucleus of *Euplotes patella* is essential to the life and proliferation of this ciliate. Organisms deprived of the micronucleus could neither live more than a few days nor divide more than twice. In two instances the micronucleus was drawn into a micropipette and returned shortly thereafter to its original position. In each instance the organisms survived and formed a vigorous colony. Thus, the micronucleus was able to survive in the micropipette for a short time. Undoubtedly it had been surrounded by some cytoplasm or cytoplasmic residue for, in the light of our present knowledge, it could not have come in contact with the external fluid environment.

Transplantation of cytoplasm. Duryee² produced typical nuclear radiation damage in nonirradiated eggs of *Triturus viridescens* by microinjecting cytoplasmic material extracted from X-irradiated eggs into the cytoplasm. The changes began to develop gradually 10 to 30 min. following the transplanta-

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tion of cytoplasm. The amount of cytoplasm from an irradiated oöcyte required to produce radiation damage in a normal nucleus was approximately $0.1 \mu\text{l}$. (100 cubic microns).

Daniels³ injected cytoplasm from normal giant amoebas, *Pelomyxa illinoisensis*, into X-irradiated pelomyxas. The mean survival time was significantly prolonged by injections of normal cytoplasm. The amount of cytoplasm, ranging from 1/15th to 1/10th of the volume of the cell, was enough to permit some of the animals to live following moderate exposure to X rays. Without such treatment the cells would have died.

Transplantation of the nucleus of the Amoeba. During the early development of micrurgy, many investigators attempted to transplant the nucleus from one amoeba to another, but without success. In 1939, Comandon and de Fonbrune¹⁻⁵ devised a procedure for accomplishing this interesting operation; this involved pushing a nucleus with a blunt microneedle from one cell to another without permitting the nucleus to come in contact with the external environment. Even momentary contact with any external medium instantly kills the nucleus. In these experiments, the donor and host amoebas were of the same species, *Amoeba sphaeronucleus*.

Lorch and Danielli⁶⁻⁸ and Danielli⁹ transplanted the nucleus from *Amoeba proteus* to *Amoeba discoides*, and vice versa. Most important was their report that occasional clones were derived from cells with a grafted nucleus, thereby suggesting that a successful blending of heterospecific nucleus and cytoplasm had occurred. It is interesting to note that the cytoplasm seemed to dominate the cell type, that is, a cell consisting of *discoides* cytoplasm and a *proteus* nucleus resulted in organisms showing a nuclear size and cell form characteristic of the *discoides* species. A *proteus* cytoplasm with a *discoides* nucleus produced an amoeba with a form characteristic of that usually associated with the *proteus* species. However, reference to Chambers¹⁰ criticism of these experiments should be made.

Ord and Danielli¹¹⁻¹² and Ord¹³ used the technique of nuclear transplantation to localize the action of nitrogen mustards or X radiation on *Amoeba proteus*. Their experiments showed that both the cytoplasm and the nuclei of the amoebas were damaged by large doses of X rays; the nuclei were about 2.4 times more sensitive than the cytoplasm. Furthermore, their results showed that a normal nucleus becomes damaged when transplanted into cytoplasm that has been previously exposed to X radiation.

Recently, Goldstein and Plaut¹⁴ devised experiments to demonstrate that cytoplasmic ribonucleic acids (RNA) are synthesized by the nucleus in *Amoeba proteus*. In these ingenious experiments, amoebas were fed on *Tetrahymena pyriformis* cultured in media containing radiophosphate (P^{32}). Through autoradiography the accumulation of radiophosphate in the nucleus of the amoeba could be demonstrated clearly. After radiolabeling, the nuclei were transplanted to normal amoebas, using the technique of de Fonbrune.⁵

On fixing amoebas five hours after the operation, the autoradiographs showed that essentially all radioactivity was still localized within the nucleus. Little or no radioactivity was evident in the cytoplasm. Amoebas fixed twelve or more hours after the transplantation, however, showed appreciable

radioactivity in the cytoplasm, with diminishing radioactivity in the grafted nucleus. These experiments were interpreted to indicate that radiolabeled RNA, or some derivative of RNA, was transmitted from the grafted nucleus to the cytoplasm. Prior treatment of the amoebas with ribonuclease (RNase) abolished radioactivity in both the nucleus and the cytoplasm, thus confirming the interpretation that the RNA fraction was involved in the shift from nucleus to cytoplasm. There was no transfer of radioactivity (presumably radio-RNA) from the cytoplasm to the nucleus.

Transplantation of amphibian nuclei. Briggs and King¹⁵⁻²⁰ have transplanted nuclei from cells of frog embryos into the cytoplasm of enucleated frog eggs, mainly of *Rana pipiens*. This was accomplished by drawing a cell containing the donor nucleus into a micropipette. Some of the cytoplasm would be torn away during the transfer, but the cytoplasmic residue apparently was sufficient temporarily to protect the nucleus from artificial media. Without such protection, the nucleus would be killed before transplantation could be completed. It is noteworthy that the larger the cell containing the donor nucleus, the better the chances of achieving successful transplantation. Accordingly, transplants of nuclei from blastula cells were more apt to be successful than were those obtained from gastrula cells.

Despite the inherent difficulties of protecting a nucleus with a relatively small amount of cytoplasm, it was possible to transplant the nuclei from cells of presumptive chorda and medullary plates into the enucleated frog eggs. As a result of the transplant, the eggs cleaved normally and developed into a complete blastula, with some continuing onto the neurula or post-neurula stages. There were no significant differences produced between the two classes of donor nuclei, even though one is destined normally to differentiate into mesodermal organs and the other to neural organs.

Experimental transposition of nucleoli. A few examples of experimentally transposed nucleoli in frog tumor cells (Lucke adenocarcinoma of the kidney) and also in cells from human ovarian neoplasm have been studied by newly devised techniques.²¹ Initially the experimentally transposed nucleoli were small and compact, and in each instance the entire nucleolus was transposed. The following changes were seen. In one there was an enlargement with a suggestion of disintegration while, in another, obvious fragmentation was seen. In others there was the appearance of extensive vacuolization. On the other hand, following transposition some nucleoli showed no detectable changes within the time limits of observation. All observations mentioned here were made under bright field illumination with the television microscope.

These are preliminary explorations designed primarily to test the practicability of the various transplantation procedures devised for this work. The main difficulty is the maintenance of the cells in a viable state for periods ranging from several hours to several days, so that enough time may be allowed for all possible changes, either in the nucleolus or in the cytoplasm, to occur.

For short-term experiments the effects of transplantation on either the nucleolus or its new environment are being evaluated with the television-micromanipulator-oscilloscope unit, whereby single scan lines selected from

the video image are displayed as densitometric curves on the screen of a fast-rise cathode-ray oscilloscope.²¹ This procedure is useful in connection with the supravital staining of the nucleoli by methylene violet (Bernthsen).

Improvements in Subcellular Transplantation

Micrurgy with cells in nonaqueous media. A new approach to the transplantation of subcellular particles has been developed; this requires the transfer of cells from their normal aqueous environment to a nontoxic fluorocarbon fluid.²² Protection to the subcellular structures during removal and subsequent transfer is provided by sealing the sample within the micropipette, at both ends, with small volumes of polymerized trifluoromethylchloroethane. The micropipettes are coated with a silicone to prevent coagulation of the cytoplasm or other cellular fractions, and also to prevent the subcellular particles from adhering to the glass. The fluorocarbon seals protect the sample from chance contamination with, and subsequent injury from, aqueous media.

Nucleoli can be removed from the nucleus of one cell and placed into either the nucleus or cytoplasm of another cell with siliconized micropipettes. Micropipettes with small apertures, 1 μ or less, can be used for removing pieces of the nucleolus. In a similar manner, the inclusion bodies from either the nucleolus, the nucleus, or the cytoplasm can be transferred with a micropipette to any part of the same or different cells.

Instrumentation. An important advance in micrurgy is the volumetric submicromanipulator for removing or implanting known volumes of subcellular substances into living cells.^{23, 24} Another advance is the television micromanipulator, using the RCA closed-circuit television. The television micromanipulator has now been augmented with a Conrac 17-in. auxiliary viewer and a fast-rise cathode-ray oscilloscope (FIGURE 1). The oscilloscope, Tektronix type 545 with the 53, 54C dual trace preamplifier, has a flexible sweep-delay circuit. Virtually any one of the horizontal lines comprising the video image can be selected and displayed as a densitometric trace on the oscilloscope. At the same time, a pulse from the positive gate is fed into the viewer to blank out, and thereby indicate, the line selected on the video screen. Four Leitz micromanipulators and two semiquantitative volumetric controls are used to perform the routine transplantation operations on cells.

A photograph of a transplanted nucleolus, together with the oscilloscope traces of three selected lines, is shown in FIGURE 2. Using the method of micropipette transfer, the nucleolus was transplanted from one frog kidney tumor cell, in tissue culture, to another kidney tumor cell. The preparation was fixed in cold methanol and was stained with methyl green and pyronine Y approximately 12 hours after the nucleolus was transplanted. The photograph of the cell was taken from the image on the video screen.

Design of experiments. The notation of symbolic logic and calculus of sets was first applied to the analysis of the properties of nucleoli.^{21, 25} This scheme has now been revised so that common symbols can be employed. The following have been selected: *n*, nucleolus; *N*, nucleus; *C*, cytoplasm; *e*, element of; *g*, subgroup of; $-$, not or excluding; *v*, join; and *x*, intersect.

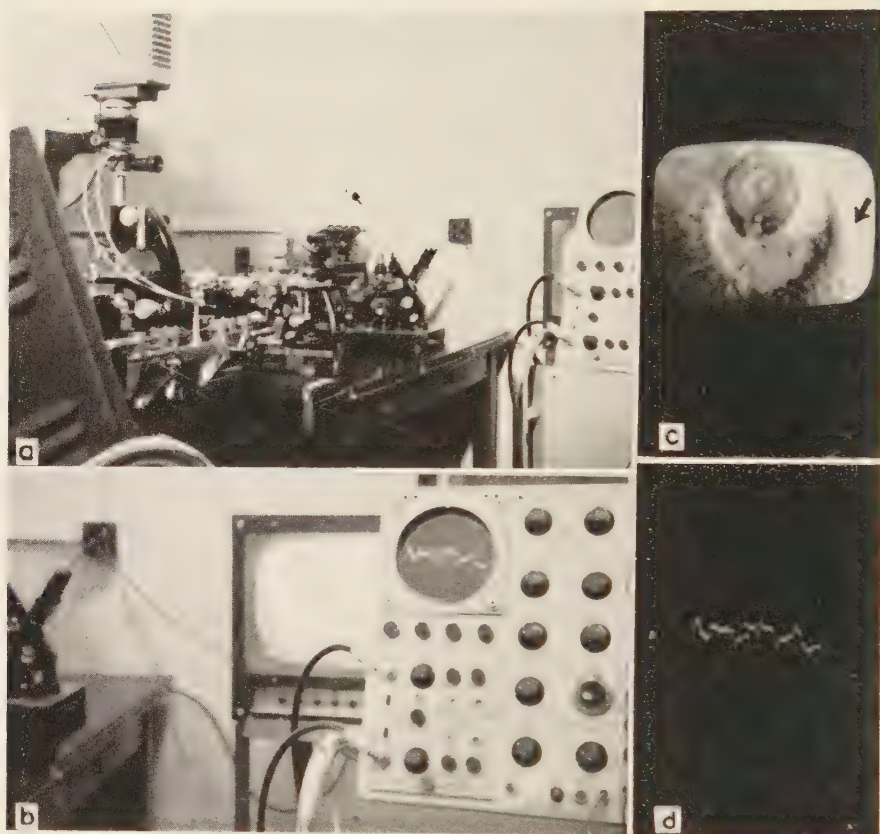


FIGURE 1. Television-micromanipulator-oscilloscope unit. FIGURE 1a shows the Vidi-con camera mounted on a special stand together with the micromanipulators and semi-quantitative volumetric microinjectors. The monitor and control for the camera are seen at the extreme left side of the picture. The oscilloscope is shown at the right side, with the auxiliary viewer mounted behind it. Other micromanipulators, including the volumetric submicromanipulator, are shown on the table between the television unit and the oscilloscope. FIGURE 1b shows the oscilloscope and the auxiliary viewer. Note the tracing of the selected television line on the screen of the oscilloscope. FIGURE 1c is a picture taken off the screen of the auxiliary viewer, showing a section of an immature human oocyte surrounded by follicle cells. The black horizontal line (arrow) that transects the oocyte below the nucleus indicates the television line selected for simultaneous analysis on the oscilloscope. FIGURE 1d shows the tracing of the selected line on the oscilloscope. The information is presented as an absorption curve, that is, the higher the pulse level, the higher the absorption of light by the part of the cell scanned; similarly, the lower the pulse level, the higher the transmission of light.

When two or more elements are a subgroup and are not in contact, they are designated by *v*. However, if the elements are in contact with one another, then *x* is used. The letter *Q* designates quantity. For example, *Qn* from *n*, signifies a part of a nucleolus, while (*Qn*) from (*n*) means one nucleolus from several nucleoli present in a multinucleolate cell. Furthermore, (*Qn e N*)

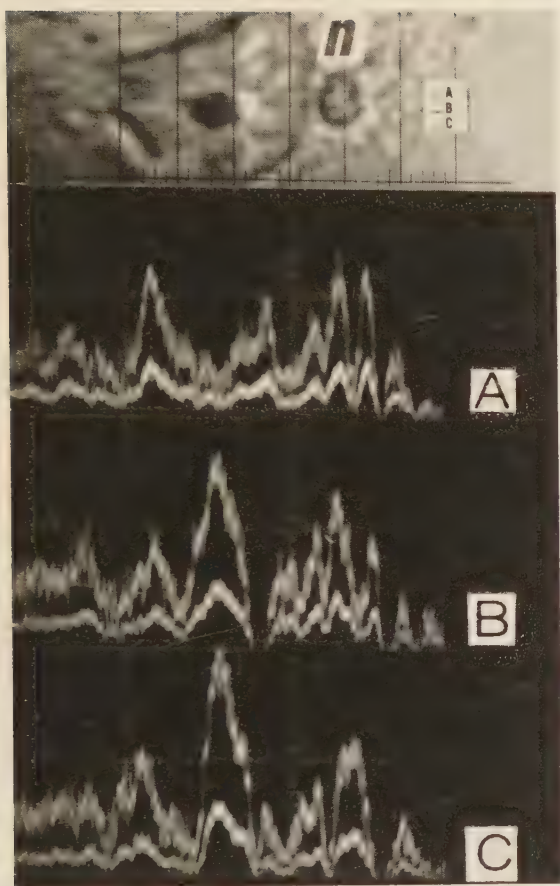


FIGURE 2. Video picture of a transplanted nucleolus. This photograph shows the nucleus of a frog kidney tumor cell, in tissue culture, with a dense, centrally positioned nucleolus (between the second and third vertical lines). The structure below the label *n* is a nucleolus transplanted from another tumor cell growing in the same culture. This nucleolus shows a prominent central vacuolar zone and is surrounded by a well-defined chromophobic corona. The three video lines selected for analysis appear as black horizontal lines in the photograph and are labeled A, B, and C. The oscilloscope traces are shown below with the letters corresponding to the selected lines. The traces were positioned and the sweep time was adjusted so that the key vertical portions of the traces registered reasonably well with the structures shown in the cell. The dual trace pre-amplifier unit was used, with one amplifier set at a high level (upper trace) and the other at approximately one third of the high level (lower trace). The pulse amplitudes rose higher at higher amplification, however, and so also did the noise levels.

Note the high pulse amplitudes in B and C, which represent the optical density of the nucleolus *in situ*. The pulse height is proportional to light absorption. Trace A shows profiles (two peaks) of both the margin and large central vacuole of the transplanted nucleolus. The maximum absorption by the transplanted nucleolus is roughly one half of the maximum absorption shown by the nucleolus *in situ*. The chromophobic corona appears on the trace as sharp dips on either side of the two peaks produced by the nucleolar margin.

The cell was photographed (triple exposure to show the position of each selected line) from the video screen with a Polaroid camera while the oscilloscope traces were recorded with the Aremac automatic Recordscope.

denotes the remainder of nucleolar substance after Q is taken out, while $(Qn\ g\ N)$ indicates the remaining nucleoli after one or more are taken out.

The subscripts are used to identify the cell. If the subscripts before and after the operation are the same, then the operation is transposition; if the subscripts are different, the operation is transplantation. Thus, $n_1\ e\ N_1\ g\ C_1$ to $n_1\ e\ C_1 - N_1$ states that the nucleolus was transferred from the nucleus to the cytoplasm of the same cell, and the operation is transposition. On the other hand, $n_1\ e\ N_1\ g\ C_1$ to $n_1\ e\ C_2 - (n_2\ e\ N_2)$ indicates transplantation, since the nucleolus from cell 1 is transferred to the cytoplasm of cell 2. The statement further indicates that cell 2 still has its own nucleolus.

Two other examples are illustrated in FIGURE 3. In 3a the nucleolus was first transposed from the nucleus to the cytoplasm. Later a nucleolus from another cell was transplanted into the cytoplasm. Thus, $n_2\ v\ n_1\ g\ C_1 - N_1$ shows that the nucleolus from cell 2 (transplantation) and the nucleolus from cell 1 (transposition) are now a subgroup of the cytoplasm of cell 1. This is a method devised for comparing the effects of transplantation and transposition on nucleoli as well as on the cytoplasm. In this example both nucleoli are exposed to the same cytoplasmic environment, although the cytoplasm may be subjected to the action of either two similar or possibly two different nucleoli. Transposition experiments, therefore, serve as controls for transplantation experiments. This is the most direct way of controlling and evaluating the effects of genetic constitution on either the nucleolus or the action of the nucleolus on its new environment. It is to be noted that the notation also indicates the order of the operation; if the statement read $n_1\ v\ n_2\ g\ C_1 - N_1$, it would signify that nucleolus 2 was transplanted before nucleolus 1 was transposed.

Many variations of transposition and transplantation experiments involv-

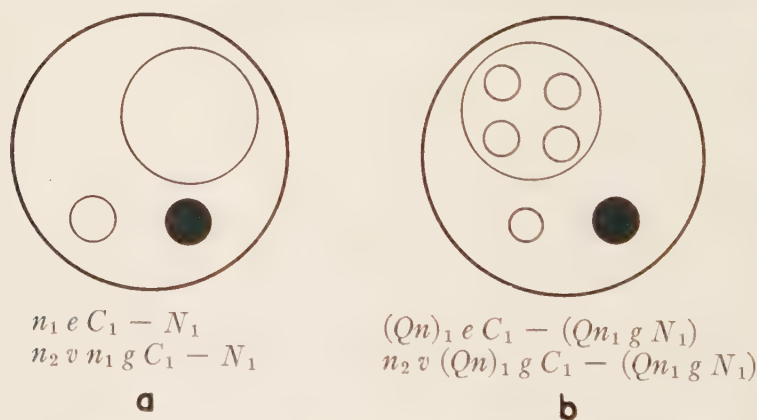


FIGURE 3. Diagrams of transplanted nucleoli with their symbolic notations. FIGURE 3a shows one transposed nucleolus (white) and one transplanted nucleolus (black) in the cytoplasm. This system is used for comparing the effects of transposition and transplantation on the nucleolus. FIGURE 3b shows one of five nucleoli (white) transposed and one transplanted nucleolus (black) in the cytoplasm. The meaning of the symbols is explained in the text.

ing nucleoli are possible, and each operation, using symbolic notation, may be precisely stated. The source and position of the nucleolus prior to the operation, as well as its position following the operation, are always recorded. For example, in FIGURE 3b, $((Qn_1) \in C_1 - (Qn_1 \text{ g } N_1))$ states that one nucleolus, (Qn_1) , from a multinucleolate cell was transposed to the cytoplasm, while the remaining nucleoli are still in the original nucleus. The second statement, $n_2 \text{ v } ((Qn_1) \text{ g } C_1 - (Qn_1 \text{ g } N_1))$, signifies that a nucleolus from cell 2, following the first transposition operation, was transplanted to the cytoplasm of the same cell.

This scheme has also been applied to the inclusion bodies found in cells of the frog renal adenocarcinoma. These inclusions, which are frequently eosinophilic, may be found in nucleoli, in the nucleus, or in the cytoplasm. Duryee^{26, 27} has concluded that the substance is first elaborated by the nucleolus, and from here the inclusion material may pass into the nucleus and then into the cytoplasm. Our observations have confirmed Duryee's conclusions and have extended them to include cells obtained from certain human ovarian neoplasms.

The inclusion body is designated by the symbol b . The intranucleolar body is $n(b)$. Thus, $n(b) \in N \text{ g } C$ states that the inclusion body, enclosed by the nucleolus, is an element of the nucleus. An intranucleolar body that becomes partly extruded into the nucleus may be designated by either $b \text{ x } n$ or by (bn) . The former implies an intersection of inclusion with the nucleolus, while the latter considers the body and nucleolus as a unit. There are two ways of indicating a nuclear inclusion body: (1) $b \in N - n \text{ g } C$ or (2) $b \text{ v } n \text{ g } N \in C$. The former states that the body is an element of the nucleus, but not of the nucleolus, while the latter states that the body and nucleolus are independently a subgroup of the nucleus. If, however, there is any contact between the body and the nucleolus, it must be designated by $b \text{ x } n$ or (bn) .

When partly extruded into the cytoplasm, the intranuclear body becomes $b \in C \text{ x } N - n$, which means that the body is an element of the cytoplasm, while also intersecting with the nucleus, but not with the nucleolus. Finally, the cytoplasmic inclusion body becomes $b \in C - (n \in N)$; thus the body is an element of the cytoplasm, but not of the nucleus or any of its components. The same situation may also be stated $b \text{ v } (n \in N) \text{ g } C$, meaning that the body and the nucleus, with its nucleolus, are independently a subgroup of the cytoplasm. If the inclusion body was attached to the nucleus it would be designated by the intersect x .

The symbolic statements are most useful in setting up important combinations for establishing the relationship of the inclusion body to the formation of nucleolar lesions.²¹ We are attacking this problem by using the methods of transposition and transplantation on either nucleoli or inclusion bodies or both. Some fifty combinations, involving the origin of the inclusion body and of the nucleoli, can be made. Two such combinations are indicated in FIGURE 4.

In FIGURE 4a, a nucleolus from cell 1 is transplanted to the cytoplasm of cell 2, which contains a cytoplasmic inclusion body. In this situation the

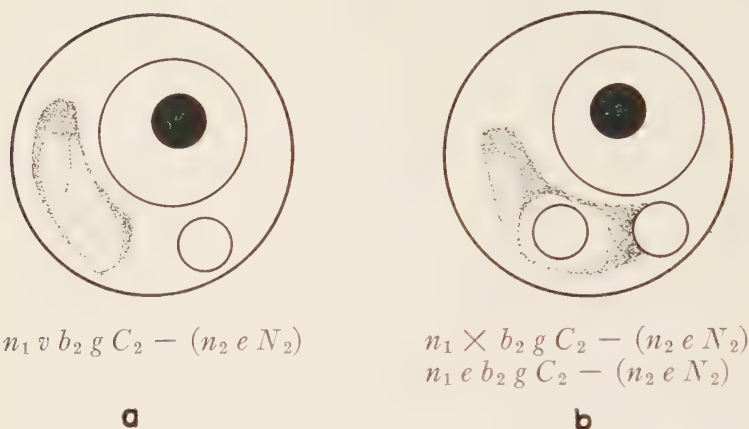


FIGURE 4. Diagrams of transplanted nucleoli in cells containing cytoplasmic inclusion bodies, with symbolic notations. In both diagrams the inclusion body is stippled. FIGURE 4a shows a nucleolus, transplanted from another cell, placed into the cytoplasm without coming in contact with the inclusion body. FIGURE 4b shows two types of nucleolar transplantations. The difference is in the positions of the nucleolus in relation to the inclusion body. In one, the nucleolus is touching the body; in the other, the nucleolus is embedded in the inclusion substance. The meaning of the symbols is explained in the text.

transplanted nucleolus and the inclusion body are not in contact. In FIGURE 4b, two additional types of transplantation are shown. Here again the host cell contains a cytoplasmic inclusion body. In one instance, $n_1 \times b_2$, the nucleolus is transplanted to a position that establishes an intersect, or direct contact, with the body. In the other, $n_1 e b_2$, the nucleolus is transplanted directly into the body, and thus has become an element of that body.

In a hypothetical series, cell 1 is the normal cell, cell 2 is a neoplastic cell with an inclusion body, and cell 3 is a normal cell. The first operation, $n_1 e N_1 g C_1$ to $n_1 e C_3 - (n_3 e N_3)$, is the transplantation of a nucleolus from cell 1 to the cytoplasm of cell 3. The second operation, $Qb_2 e N_2 - n_2 g C_2$ to $Qb_2 v n_1 g C_3 - (n_3 - N_3)$, states that part of the inclusion body from the nucleus of cell 2 (intranuclear inclusion) was transplanted into the cytoplasm of cell 3, so that the inclusion body from cell 2 and the nucleolus from cell 1 are independently a subgroup of the cytoplasm of cell 3. An experiment of this type will show not only the action of the inclusion substance on a transplanted nucleolus, but also the action on the nucleolus of cell 3, *in situ*. On the other hand, if the second proposition states that $b_2 \times n_1 g C_3 - (n_3 e N_3)$, then this would indicate that the inclusion body and the nucleolus from cell 1 are in direct contact. If a part of the inclusion material was transplanted, this would be designated by Qb .

The hypothetical production of experimental nucleolar lesions²¹ by transplantation is further indicated by the following statements. For example, if, following transplantation of b into cell 3, there is an increase in inclusion material, then the statement would become $(Qb_3 v b_2) v n_1 g C_3 - (n_3 e N_3)$, where $(Qb_3 v b_2)$ denotes an increase of inclusion substance, with b originating

in cell 3. If the inclusion substance should grow and incorporate the nucleolus, then this would be indicated by $Qb_3b_2(n_1) \text{ } g \text{ } C_3 - (n_3 \text{ } e \text{ } N_3)$. On the other hand, if the nucleolus were to start elaborating inclusion substance, this would be indicated by $n_1(b_3) \text{ } g \text{ } C_3 - (n_3 \text{ } e \text{ } N_3)$. In this instance, $n_1(b_3)$ indicates a nucleolar lesion. The development of inclusion material in the nucleolus of cell 3 would be indicated by $b_2 \text{ } v \text{ } n_1 \text{ } g \text{ } C_3 - (n_3(b_3) \text{ } e \text{ } N_3)$.

The hypothetical generation of nucleolar lesions may be further indicated by the following statements. In general, the nucleolar lesion may be designated by the symbol *nl*. Thus, $n_1 \text{ } e \text{ } N_1 \text{ } g \text{ } C_1$ to $nl_1 \text{ } e \text{ } N_1 \text{ } g \text{ } C_1$ states that the normal nucleolus becomes spontaneously transformed into a nucleolar lesion. The next, $nl_1 \text{ } e \text{ } N_1 \text{ } g \text{ } C_1$ to $Qnl_1 \text{ } v \text{ } (n_2 \text{ } e \text{ } N_2) \text{ } g \text{ } C_2$, states that part of the nucleolar lesion is transplanted into the cytoplasm of cell 2. If the normal nucleolus in cell 2 forms a nucleolar lesion, then this would be represented by $Qnl_1 \text{ } v \text{ } (nl_2 \text{ } e \text{ } N_2) \text{ } g \text{ } C_2$, which states, in general, that a new nucleolar lesion was produced. By appropriate choice of symbols, as discussed previously with the inclusion bodies, the type of nucleolar lesion could be designated with still greater precision.

Problems Requiring Solution

Tissue cultures for micrurgy. The complete evaluation of biological effects, following nucleolar and other transplantations, is contingent on the survival of cells following the operations. Except for the more instantaneous responses of either the nucleoli or the cytoplasm following transplantation, the only means of determining the action of nucleoli on the new cytoplasm, or vice versa, is through the subsequent behavior of the modified cells. This is especially important also, since certain biological effects may not appear until the next generation of cells. In most instances the approach to the maintenance of cells is through modifications of tissue-culture procedures.

Although several methods for the production of clones from isolated single cells have been described in recent years, none seems to be sufficiently practicable to be of value where the cells have been subjected to prior micrurgy.

Micrurgy and tissue culture have not, as yet, been perfectly blended. One deterring factor is the present unavailability of a chamber satisfactory for use for both micrurgy and tissue culture. One attempt to solve this problem was mentioned recently by Crocker *et al.*,²⁸ using the oil "coverslip," or chamber, of de Fonbrune,⁹ through which a microneedle or micropipette can be passed. The growth of cells in tissue culture under oil has been reported by Ludford.²⁹

The approach used by my associates and myself now includes the development of a radically different culture vessel that will satisfy all requirements for tissue culture, including both solid and liquid media, and also all requirements for micrurgy. Primarily, it is also necessary to permit micrurgy in the tissue-culture chamber without unnecessarily disturbing the cells growing in the culture. With this arrangement, it should likewise be possible to avoid further disturbance of the host cells following transplantation.

Other requirements for cells to be used for transplantation studies follow. The donor cells should be flattened, since it is much easier to see and thereby to obtain the desired structure from that cell with a micropipette. The recipient or host cells, however, must be essentially spherical in order to present a better target, in depth, for implanting the subcellular particles. To satisfy these two requirements, the culture chamber must provide a means of culturing cells in both fluid and semisolid culture media simultaneously. In general, cells show a tendency to "round up" in fluid media, while solid media promote flattening.

Even though single-cell isolation and culture is still impracticable, especially with cells subjected to extensive microsurgery, consideration is given to the procedures described by Puck *et al.*³⁰ and Marcus *et al.*³¹ in reference to the "feeder layer" that seems to work unusually well in aiding the growth of relatively few cells derived from the HeLa strains. A small group of cells (especially if they form a recognizable pattern) can be identified and subsequently reidentified. Such a configuration of cells could readily serve as host cells, especially if they are rounded up. Morphological changes in such cells, following transplantation of subcellular particles, can be followed best by time-lapse or sequence photography (15 to 30 min. between pictures), as suggested by Duryee.^{26, 27} Motion picture cameras must be used for time-lapse records, while conventional photomicrographic cameras are more suitable for sequence photography.

Sterility, especially of the micropipettes, offers no serious problems at present. The micropipettes can be sterilized conveniently by short exposure to high-intensity ultraviolet light. The culture medium usually contains penicillin or other antibiotics, so that sterilization of the micropipettes is not absolutely required.

Microinjection procedures. The removal of a nucleolus from its normal nuclear site presents special problems. One of these involves a means of introducing suction at variable intensities over limited, although adjustable, volumes. This requirement, therefore, necessitates something in addition to the presently available facilities for microinjection, including micrometer-controlled syringes²⁴ and the volumetric submicromanipulator.^{22, 24} One possibility now being tested is the combination of present methods (micrometer-controlled syringe) with a magnetostriction-driven piston for regulating both the volume range and the speed of attaining such volume changes. The point to be emphasized is that slow suction must be applied to pull the nucleolus on its way into the orifice of the micropipette. Then, at the proper moment, suction must be applied vigorously in order to extract the nucleolus from its nuclear position while, at the same time, the total volume of material brought into the micropipette must be carefully regulated. An electrically controlled drive seems to offer the most promise, especially since drives of this type can be readily programmed for semiautomatic and automatic operations.

Instrumentation. A new four-place micropositioner is now being constructed (Harris and Kopac³²). Included in the design is an automation control unit whereby certain micurgical operations such as positioning of

the micropipette with reference to a nucleolus can be programmed and, hence, automatically performed. Included with this instrument is an entirely new inverted microscope combined with the flying-spot principle (for visible and ultraviolet light) plus a remotely controlled fine adjustment and mechanical stage. The fine adjustment through automatic programming alternately will maintain two different levels in focus. Scan-line selection and electronic computing devices will be used with this instrument to analyze changes in nucleolar structures and their physicochemical properties following transposition or transplantation. The new micropositioner and microscope can also be controlled manually, but motion will be induced and controlled by push buttons, rather than by the levers, control knobs, or other schemes used heretofore. The magnetostrictive volume control for the micropipettes will be integrated with the micropositioner for both manual and subsequent automatic operation.

Significance of Subcellular Transplantation Studies

Through various developments in micrurgical techniques, particularly in those that make subcellular transplantations feasible, a direct attack on the properties and functions of nucleoli is possible. The nucleolar lesion may now be studied experimentally, as can the inclusion bodies of human ovarian neoplasms and those seen in the cells of the Lucke tumor.

Means are now available for establishing the existence of the nucleolar-chromosomal complex as one of the basic regulatory mechanisms in the cell. If this complex regulates not only nucleolar growth and function, but chromosomal synthesis as well, it is possible that the regulatory mechanism can be upset by modifications or dissociation of the nucleolar-chromosomal complex. Following such dissociation, will nucleolar synthesis now become more or less independent of other cellular activities? Will chromosomal synthesis, especially that normally associated with nucleoli, also become independent? Will such independence lead to the abnormal production of DNA? What effects would such changes have on other cellular activities? Can the uncoupling between the nucleolus and the chromosome be induced by the action of certain viruses? These are some of the questions now facing the investigator.

Although primary focus is on the role of the nucleolus in the cancer process, as determined by applying transplantation techniques, the same experimental and theoretical procedures are directly applicable to several problems in connection with the properties and functions of other subcellular particles. Of these, three are singled out for special consideration: (1) chromosomal transplantation, (2) transplantation of cytoplasm from melanocytes to amelanocytes, and (3) transplantation of nuclear and cytoplasmic inclusion bodies in the cells of the renal adenocarcinoma of the frog to normal kidney cells. The latter aspect is also being investigated competently by Duryee.

Finally, if aberrant mitochondria are involved in the neoplastic processes, as suggested by Warburg,³³ then it should be possible to induce the condition in normal cells by inoculating them with mitochondria derived from neoplastic cells. If Warburg is correct, the "damaged" mitochondria should

reproduce in an appropriate cytoplasmic environment such as might be provided by transplanting the mitochondria into normal cytoplasm of genetically related cells. Experiments of this type should help clarify the issues concerning the role of mitochondria both in the cancer process and in respiration. The latter property, believed significantly to be impaired in neoplastic cells, definitely requires clarification. Certainly, critical experiments with properly executed mitochondrial transplants should supply answers to our questions that the colloquia recently published by Weinhouse³⁴ and by Warburg³⁵ and Burk and Schade³⁶ unfortunately are unable to furnish.

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Part III. Viruses in Neoplastic Tissue

LATENCY OF INDUCED VIRUS INFECTION

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A discussion of all the many ways in which the term "latency" is used would be far beyond the scope of this presentation. Instead, only a few meanings will be discussed in the light of some of the newer techniques that have made possible better methods of studying virus-cell relationships. Indeed, if some of the present hopes are fulfilled, the animal virologists will be in the enviable position of the bacteriophage workers.

The first meaning of the word to be considered is that used to describe the stage of virus multiplication that follows adsorption and lasts until newly formed virus is released. In other words, no infectious particles per se are detectable, and it is only by subsequent events that one is able to tell that virus had been present. This interval is also called the eclipse phase, or the dark period or, when the originally infecting virus is not removed from the system, the constant period.

The growth cycle has now been worked out for a number of virus-cell combinations; and the results can be examined with the object of finding out what factors influence the latent period. It must be emphasized that the data, presented only to illustrate certain points, are by no means complete, and that, indeed, in some instances the information has been taken from experiments planned for other purposes. Some of the results found for the influenza virus are summarized in TABLE 1.¹⁻⁵ It is apparent that the latent period varies considerably. The variations are certainly a reflection of the different methods by which the period was determined. For example, when determined in the fertile egg for influenza A, it is 5 to 6 hr.; in the de-embryonated egg it is 4 to 5 hr.; in minced chorioallantoic membrane it is 2 to 3 hr. One can surmise that these differences also are a reflection of the influenza virus's ability to be adsorbed on various cell surfaces, although measures were taken to minimize this possibility. Another factor that influences the result is illustrated by the work of Cairns,² in which different amounts of virus were inoculated into embryonated eggs, showing that the latent period was lengthened when less virus was inoculated. This has been found true for other virus-host cell systems. It also appears that influenza B has a longer latent period than the A strain.

Some of the reported results on the different strains of poliovirus are presented in TABLE 2.⁶⁻¹¹ All of these experiments have been done in isolated systems, with removal of virus after infection, replacement of growth media, and then a recording of the elapsed time before the appearance of new virus. There are explanations for some of the differences noted. For example, Lwoff *et al.*⁶ point out that their relatively long latent period ($5\frac{1}{2}$ to $6\frac{1}{2}$ hr.),

TABLE 1
LATENT PERIODS OF INFLUENZA VIRUS

Author	Virus strain	Host system	Latent period (hours)
Henle & Rosenberg ¹	A	Fertile egg	5-6
	B	Fertile egg	8-10
Cairns ²	A	Fertile egg inoculated with:	
		800 AD*	4
		73 AD	5
		14 AD	6-8
		4 AD	10
Finter <i>et al.</i> ³	A	De-embryonated egg	4-5
Tamm & Tyrell ⁴	B	Whole chorioallantoic membrane in tissue culture	5½
Ackermann & Francis ⁵	A	Minced chorioallantoic membrane	2-3

*. Agglutinating doses.

TABLE 2
LATENCY PERIOD OF POLIOVIRUS

Author	Strain of virus	Cell system	Latency period (hours)
Lwoff <i>et al.</i> ⁶	Type I	Monkey kidney (single cell)	5½-6½
Dunnebacke ⁷	Types I, II, III	Monkey kidney	6
Harding <i>et al.</i> ⁸	Type II	HeLa	6
Ackermann & Francis ⁵	Type III	HeLa	4-5
Reissig <i>et al.</i> ⁹	Types I, II, III	Monkey kidney	4
McAllister <i>et al.</i> ¹⁰	Types I, II, III	Chang conjunctiva Chang kidney Monkey kidney	4-8
Fogh ¹¹	Type II	HeLa Monkey kidney	3½ 5½

obtained by a study of single cells, may have been due to selection (since only those cells that adhered to glass were studied) or due perhaps to the temperature change that occurred during manipulations. Fogh¹¹ points out that his short latent period was obtained with a high multiplicity of infection. Certain factors do not seem to be involved in the results. For instance, all

SEMLIKI VIRUS

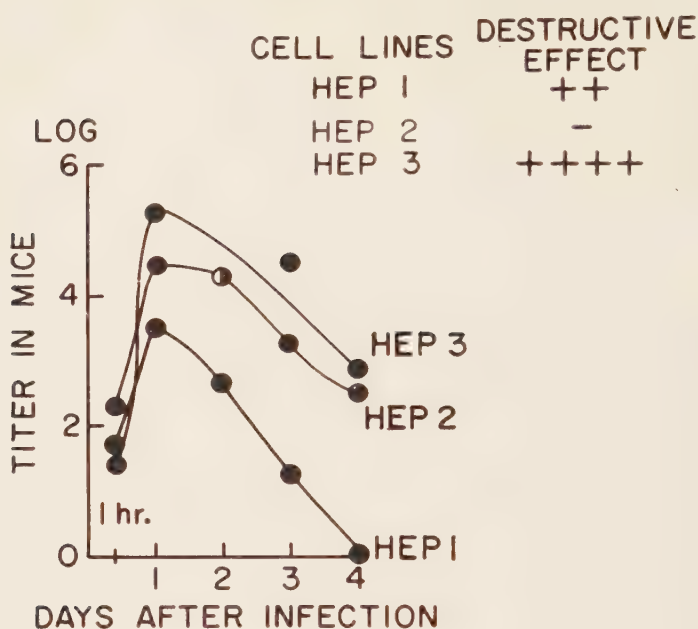


FIGURE 1

types of poliovirus have approximately the same length latent period and, whether monkey kidney, HeLa cells, or Chang's cells are used as the cellular component, the results are comparable. McAllister *et al.*¹⁰ have pointed out also that different types of media, whether enriched with horse or human serum, had no influence on the result. Fogh¹¹ noted no pH dependence.

By one method or another the latency period has been determined for practically all classes of viruses. The only generalization that seems safe to make is that the latent period for the larger viruses appears to be longer; for example, 20 hr. for the psittacosis group¹² and 10 hr. for vaccinia.¹³ It should be borne in mind that these viruses form inclusions; much admirable work has been done in establishing a correlation between the histopathology and the appearance of the virus. No correlation has been noted between virus size and the latent period for the smaller viruses.

Since we are interested in the effects of neurotropic viruses on various cell lines grown in tissue culture, we have studied the growth curves of some of these viruses against our different cell lines. This has been done by allowing the cells to grow in sheet form on the walls of test tubes, exposing them to large amounts of virus for 2 hr., at 37° C., and then removing the fluid, washing the cells 5 times, and adding growth media.* Titers on the

* Media for the daily growth curves consisted of beef amniotic fluid plus 5 per cent horse serum plus 5 per cent beef embryo extract. Media for hourly growth curves consisted of Eagle's synthetic media plus 10 per cent human serum.

supernatant were done 1 hr. later, and daily thereafter. FIGURE 1 gives the curves obtained when 3 human epidermoid carcinomas (HEP No. 1, HEP No. 2, and HEP No. 3)¹¹ were exposed to Semliki virus. Although the titers obtained differed, the general shape of the curve was the same whether the tumor was completely destroyed (HEP No. 3), partially destroyed (HEP No. 1), or suffered no destruction at all (HEP No. 2). We have found this to be true of other tumor cell-virus systems.

An effort was made to determine whether there was a difference in the latent period of this same virus and the 3 different cell lines. The same type of experiment was done, but at shorter intervals. In addition, the cells were removed from the surface, washed, ground with alundum, and tested for virus. Cell counts were also done. No cytopathogenicity was noted within the time of this experiment (24 hr.), and the cell counts remained approximately the same. FIGURE 2 shows the results of titration of the supernatant; it appears that the latent period with the Semliki virus and the HEP 3 was shorter than that of the other two cell lines, which were only partially or not at all destroyed. A similar result was obtained when virus was sought in the cells. The maximum increase occurred between 3 to 6 hr. for HEP No. 3, 6 to 9 hr. for HEP No. 1, and 9 to 12 hr. for HEP No. 2.

Since the same infecting dose was used and approximately the same number of tumor cells were present in each tube (except for HEP No. 2, which had 25 per cent less cells), another system was sought to substantiate the feeling that the latent period was shorter when there was an oncolytic effect. The same type of experiment was done, using 1 tumor (HEP No. 2), but 3 different viruses: Bunyamwera, which completely destroys the tumor; Egypt 101 (a strain of West Nile), which partially destroys it; and Semliki Forest Virus (SFV), which has no effect on the cells. FIGURE 3 gives the titers on the supernatant. Although the results with the Bunyamwera virus were not as

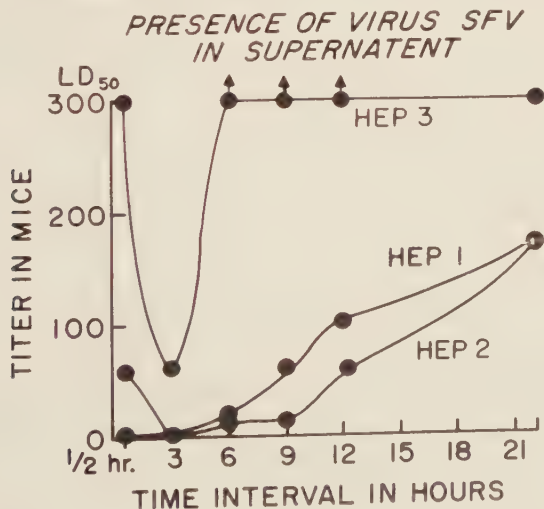


FIGURE 2

**GROWTH CURVES-HEP 2 CELLS
(SUPERNATANT)**

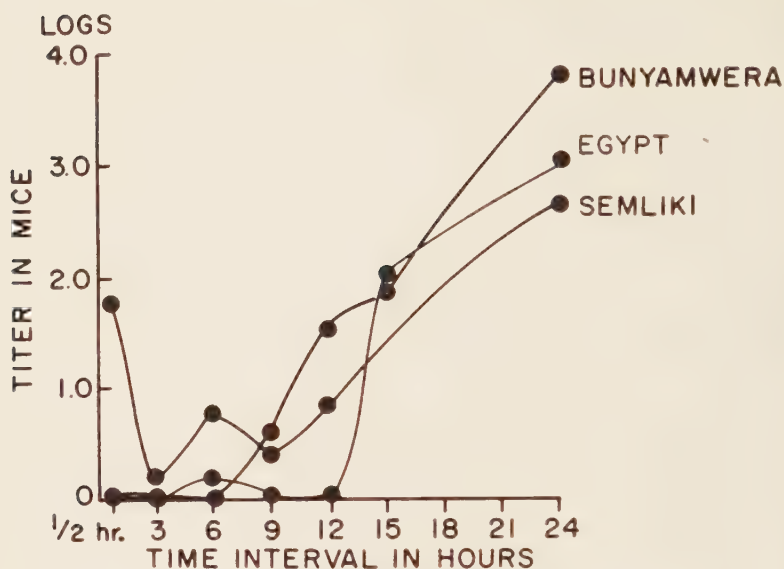


FIGURE 3

clear cut as those of the other host-virus systems, since the release of virus in the Bunyamwera-infected cells was more gradual, nevertheless it can definitely be said that its latent period was considerably shorter than that of the Egypt-infected cells, while that of the Semliki virus was between these two. FIGURE 4 shows that this also appeared to be the case when the ground cells were titered. Our general conclusion is that viruses that destroy tumor cells may have shorter latent periods, at least in these particular cell-virus systems.

A great number of environmental factors have been found to influence the length of the latency period. Morgan and his co-workers¹⁵ have described the extension of the latent period of the psittacosis-infected chorioallantoic membrane from 20 hr. to 15 days by placing them in balanced salt solution and glucose. When a growth medium was added, new virus was formed. In this way it has been possible to study the nutrients necessary for the production of virus, and these investigators have found that the water-soluble vitamins and the amino acids phenylalanine and tryptophane are necessary for viral synthesis.¹⁶

Other workers have found that varying the physiological environment has an effect on the latent period. Daniels *et al.*¹⁷ showed that influenza-infected chorioallantoic-membrane tissue cultures lacking glucose failed to support virus growth, although hemagglutinins were produced. The virus must have been in a latent form, since the addition of glucose resulted in the formation of fully infective virus. Eaton and Scala¹⁸ placed infected membranes in

hypotonic salt solution and showed an increased latent period. Up to 42 hr. after infection, this effect was reversible by restoration of the isotonicity.

The latent period can also be affected by treatment of the virus-infected host cells with chemicals. Tamm and Tyrell⁴ reported that an 80 to 100 per cent lengthening of the latent period in influenza B-infected chorioallantoic membrane preparations was produced by benzimidazole and related compounds. Using the same system, Ackermann and Maasaab¹⁹ found that α -aminosulfonic acid lengthened the latent period. The authors point out, however, that this may be due to differences in the rate of attachment of the virus; of course, in any instance where the latent period is being analyzed, one must keep in mind that latency can be increased either by slower adsorption or by slower release. In other studies these authors⁵ found that methoxinine interfered with virus multiplication in the early part of the latent period. The same group, studying the activity of fluorophenylalanine on polio-infected HeLa cells, found that the latent period was lengthened.²⁰ Aureomycin was also found to increase the latent period of psittacosis virus-infected chorioallantoic membrane from 24 to 120 hr.²¹

The latent period can be lengthened artificially by growing cells in the presence of immune serum. For example, Ackermann and Kurtz²² noted that poliovirus Type-III cells grown in the presence of immune serum fail to show characteristic cytopathogenic changes. When such cells were put in nonimmune serum for 1 passage, they still showed no virus; but when put

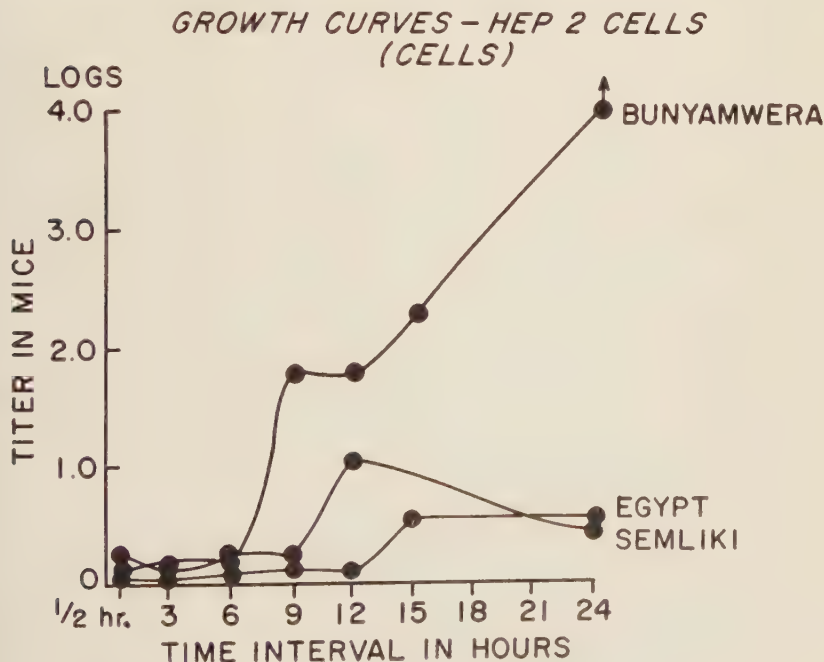


FIGURE 4

in maintenance solution, virus could be detected in 11 of 15 tests. Ginsberg and Boyer²³ also noted a protection against the cytopathogenic effect when the adenoviruses grew in immune serum, and a return of the typical destruction when the cells were put in maintenance media. We have made similar observations with two cultures of HEP No. 2 cells infected 18 and 20 months previously with Egypt 101 virus, which only partially destroys the cells. The cells put out virus indefinitely and are maintained either by trypsinization or by removing the infected fluid weekly and adding new media. In the latter instance they grow out in colonies that destroy themselves 3 or 4 days later, leaving a clump of cells that revive when trypsinized. When treated with immune serum, these cells show no cytopathogenicity.

Various procedures were tried to find the circumstances under which virus and cytopathogenicity would reappear. Cells that had been grown in immune serum were dispersed with trypsin or versene and planted in tubes with or without immune serum. Fluid was removed and replaced with growth media at 3- or 4-day intervals. In each instance, 3 fluid removals were necessary for the tubes treated with immune serum before virus reappeared, whereas the cells grown in nonimmune serum showed virus after the first fluid removal. If the cells were treated 3 or more times with immune serum, however, the virus was no longer recoverable. Where reinfected with Egypt 101, such cells were capable of supporting growth and were as susceptible to the cytopathic effect as the control cells. Also, with the idea of releasing any virus that might be contained within, they were irradiated, but they behaved like the normal cells. In this virus-cell system it appears that repeated treatment with immune serum leads to the disappearance of the virus, and our attempt to produce a model lysogenic cell has thus far been a failure.

The second meaning of the word latency to be discussed also concerns cell infection, but it differs in that the virus-host complex fails, under most circumstances, to produce new infective particles. For example, Ehrlich carcinoma cells infected with Newcastle disease virus demonstrate how temperature may influence the system to such an extent that new virus may or may not be formed.²⁴ When virus-infected allantoic fluid was mixed with the carcinoma cells at 4° or 37° C. and the mixtures were immediately inoculated subcutaneously or intraperitoneally into mice, no tumors appeared (TABLE 3). However, if adsorption had taken place at 4° C., the reaction was reversible, as shown by the fact that aliquots of these same mixtures, allowed to stand at room temperature for 1 hr. and then inoculated, readily produced tumors. We believe this to be due to the release of virus from the cells, since an increase in both hemagglutinin and infectivity of the supernatant fluid could be demonstrated. In addition to eluting spontaneously, the virus could also be removed by immune serum, receptor-destroying enzyme, or washing, as shown by tumor formation when the treated mixtures were inoculated. Indeed, the reaction resembles the hemagglutination phenomenon in most respects, except for the fact that we are dealing with a tumor cell that has its own growth potential.

TABLE 3
PRODUCTION OF TUMORS BY INOCULATION OF EHRlich TUMOR CELL-NEWCASTLE
DISEASE VIRUS MIXTURES AFTER ADSORPTION AT 4° AND 37° C.

	Tumor growth	
	Adsorption at 4° C.	Adsorption at 37° C.
Adsorption	—	—
Adsorption + elution at 25° C.	+	—
Adsorption + immune serum	+	—
Adsorption + receptor-destroying enzyme	+	—
Adsorption + washing	+	—

When the cells and virus were mixed at 37° C., the reaction was no longer reversible. The addition of immune serum or receptor-destroying enzyme failed completely to remove the virus, and the cells failed to grow when inoculated into mice. One might attribute this to growth of virus within the carcinoma cells, but when the virus was inoculated directly into an animal possessing a well-developed ascites containing ample tumor cells, viral multiplication failed to take place. In addition, large amounts of virus inoculated directly into a solid tumor resulted in the gradual disappearance of the virus. It must be present in some form in the cell, however, as shown by the fact that the *in vitro*-treated cells, when inoculated into tissue cultures of Ehrlich carcinoma cells, were capable of producing new virus, as demonstrated in our laboratory, where it has had over 100 passages, and also by Flanagan *et al.*,²⁵ who first reported its growth in tissue culture. Cells adsorbed at 37° C. and treated with receptor-destroying enzyme (RDE) or immune serum to remove virus that might have been on the cell surface were also capable of initiating infection.

More direct evidence has been obtained by Prince,²⁶ who has done fluorescent antibody studies on the virus-host system and who has been kind enough to allow me to cite some of his still unpublished results. He reports that antigen is visible as a ring around the sectioned cells after adsorption at 4° C., and the rapid disappearance of this ring at 37° C., followed by a latent period that varies in length depending on the amount of virus adsorbed, and during which no antigen is detectable. After this period the cell becomes filled with viral antigen, but shows no evidence of completely infective or hemagglutinating virus particles. It seems that this particular cell-virus system might be a fruitful one in which to study this type of latency.

In another host-cell virus system, the influenza virus and the HeLa cells, Henle *et al.*²⁷ noted that the virus produced marked cytopathogenic effect, but could not be passed in series, although there was an increase in hemagglutinins and in complement-fixing antigens. Here again the results suggest a noninfective activity, but also imply that something must be going on

inside the cell, although the formation of fully infective virus fails to take place. Indeed, there are many examples of this type of reaction in the influenza host-cell system, far too many to be given adequate attention in this paper.

So far only these two meanings of the word latency have been discussed. Both have been directly connected with the activity of the virus on the cell: in the first instance, as part of the infectious cycle, resulting in the production of new virus and, in the second instance, also as part of the infectious cycle, but without the completion of the process. We have other meanings of the word. Sometimes when special circumstances are needed to show the presence of a virus that is known to exist, the virus is termed latent. For example, at the cellular level it is possible to infect HEP No. 2 cells with the 17 D strain of yellow fever, a virus that grows well, but fails to exert any cytopathogenic effect on the cells. This particular tumor-virus combination has had eight serial trypsinizations without the appearance of cytopathogenicity or cellular change, and it is only because inoculation of the tissue-culture supernatant produces death in mice that we know that the virus exists. There is also the sad case of the "latent virus," the one that appears in the cultures or the animals, but is not the same one that had been introduced.

To summarize, I have tried to present some of the more recent findings on that period of virus infection about which we know least, with the hope that the newer techniques will make available to us much more information.

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ENHANCEMENT OF SUSCEPTIBILITY TO VIRUS INFECTION IN THE COURSE OF A NEOPLASTIC PROCESS*

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Introduction

Recently published experimental results indicate that certain viruses show an affinity for malignant neoplastic tissues, and that the majority of such neoplasms found susceptible to viral infection are transplantable into many genetically unrelated mouse strains.¹⁻³ The Ehrlich ascites tumor, which was destroyed by Bunyamwera, Mengo, and West Nile viruses,^{4, 5} the Krebs ascites,⁶ and sarcoma 37⁶ are typical examples of such genetically nonspecific transplantable tumors.

It became of interest to determine whether a tumor arising in an inbred strain of mice would also show susceptibility to viral infection and, furthermore, whether, during the process of carcinogenesis, even the early stages of the neoplastic process enhance the susceptibility of the mouse host to the viral infection.

In addition, it was planned to watch for signs of any oncolytic action of a given virus upon a chemically induced tumor. For this reason the site of application of the carcinogen became an important consideration. The uterine cervix was chosen because of its easy accessibility for intravaginal applications of carcinogen⁷⁻⁹ and viral suspensions. It was hoped that at this site virus infection would remain localized without affecting the health of the host. The progress of carcinogenesis¹⁰ and the evidence, if any, of oncolysis were determined by periodic examination of exfoliated cells in vaginal aspirates.¹¹

Material and Methods

Animals. Except in a single experiment with older mice, female virgin C3H mice 4 to 6 weeks old, obtained from the Jackson Memorial Laboratory, Bar Harbor, Me., were used throughout this study. The mice were identified, caged in groups of 5 to 10, fed standard Purina Laboratory chow, and given water ad libitum. Throughout the course of carcinogenic treatment the animals were kept in assigned quarters of the Department of Pathology at the State University of New York Downstate Medical Center. Upon completion of carcinogenic treatment the mice were transported to the Virus

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TABLE 1
ISOLATION OF MENO VIRUS FROM VAGINAL ASPIRATES
OF BENZPYRENE-TREATED MICE

Duration of carcinogenic treatment (months)	Number of virus applications	Presence of malignant cells	Virus isolation ratio of mice	
			B.P.-treated	Controls
5	8	+	6/6	0/6
7	12	+	10/25	5/25
2½	13	+	4/25	1/25
Total.....			20/56	6/56

and Research Section, Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., where they were exposed to viral infection.

Carcinogen and its application. 3,4-Benzpyrene, supplied by Edcan Laboratories, South Norwalk, Conn. was used throughout this study. A 1 per cent solution in acetone was applied routinely 2 times per week. The number of months of treatment varied according to the experiment. In the first 2 preliminary experiments (TABLE 1), painting of the uterine cervix was done with a cotton-tipped wire-loop applicator soaked in the solution of benzpyrene and introduced into the vagina until resistance was met. In the remaining experiments, including the third experiment referred to in TABLE 1, infant-size otic speculum was used to dilate the vagina, and the painting of the cervix, with the aid of the artificial illumination of a head mirror, was done under strict visual control.

In a single experiment, Tween 60, shown by others¹² to exhibit co-carcinogenic properties, was used in addition to benzpyrene in order to evaluate its effect upon the neoplastic process and upon the susceptibility to virus infection.

Preparation of vaginal smears. Vaginal aspirates were collected periodically, usually once a week, from the benzpyrene-treated mice and from the controls throughout the period of observation. Individual eye-drop pipettes with especially adapted, smooth, thin, slightly curved ends were used for each mouse. A drop of saline was first drawn into the pipette, then inserted into the vagina and, with gentle compression of the rubber bulb, expelled. By gradually releasing pressure on the bulb and simultaneously withdrawing the pipette, some vaginal fluid could be aspirated. This was immediately expelled on a clean glass slide, marked in advance with the mouse number and date. The drop of secretion was spread rapidly over the surface of the slide with the tip of the pipette, and the wet vaginal smear was submerged into the ether-alcohol in equal parts and was processed further by the standard Papanicolaou technique.

Cytologic evaluation of the neoplastic process. Results of microscopic examinations of vaginal smears were scored for the presence of 11 arbitrarily

selected cytological criteria of malignancy, the progressive number of involvements, the time of appearance, and the persistence (which had been shown¹⁰ to accompany the development of cervical carcinoma of the mouse). The criteria of malignancy were nuclear enlargement, hyperchromasia, irregular nuclear borders, prominent nucleoli, bi- and multinucleation, blue-staining cytoplasmic areas, bizarre clusters, cytoplasmic vacuolation, elongated cells, engulfment, and keratinization. They were expressed in the symbols \pm , $+$, and $++$, according to their frequency and intensity. It has been demonstrated that low-, medium-, and high-grade scores correlate with nonneoplastic cellular changes, cytological patterns characteristic of early carcinoma, and advanced carcinoma, respectively.¹⁰

Histological examination of induced tumors. Uteri of all mice that had died or had been sacrificed were saved for histological examination. Histological diagnoses were correlated with cytologic evaluations of the smears.

Viral agents tested and preparation of viral suspensions. The following viruses were used: Mengo, Bunyamwera, and vesicular stomatitis (Indiana and New Jersey strains).

Technique of treatment of mice with viruses. Upon termination of carcinogenic treatment, the mice were treated with viral suspensions by blind painting with cotton-tipped applicators inserted intravaginally. The number of applications with virus per mouse varied from 1 to 13, and the duration of the treatment period lasted from 1 to 29 days, depending on the type of experiment. In cases of multiple treatments with virus, paintings were done either on alternate days or every 2 or 3 days.

Technic of virus isolation. In cases of multiple virus treatments, vaginal swabbings were obtained either within one to several days following each virus application or after the last viral treatment. From mice undergoing single treatment with a virus, subsequent vaginal swabbings were collected 4 times several days apart.

A fresh cotton-tipped applicator was inserted, without speculum, gently into the vaginal orifice, was withdrawn and, after removal, was placed into a separate tube containing 0.3 ml. of physiological salt solution. The content of the swab was pressed out into the diluent, which then was used for the intracerebral inoculation of Swiss mice of ICR stock. The inoculum was either undiluted material or aliquots of serial tenfold dilutions made in physiological salt solution.

Mice were observed for 14 days, and the number of sick and dead were recorded daily. In most cases brain tissue was removed from one animal per experimental group, and the presence of the virus was identified by a neutralization test (see below). On many occasions virus-treated mice were exsanguinated at the termination of the experiment and their sera were submitted to a neutralization test to determine the presence of antiviral antibodies.

Neutralization tests. Dilutions of the presumably infected mouse-brain tissue were mixed in equal volume with respective immune sera and were incubated for 1 hr. at 37° C. The virus-sera mixtures were then injected either intracerebrally in mice (the first series of experiments with Bunyam-

wera, Mengo, and vesicular stomatitis viruses) or into tissue-culture tubes containing trypsinized monkey renal epithelium grown in nutrient medium No. 199. The tissue-culture neutralization test was employed exclusively in the second series of experiments for identification of Mengo and vesicular stomatitis viruses. Survival or lack of cytopathogenic effect in the tissue culture indicated positive identification of the virus.

Mouse sera (see above) were diluted with an equal volume of physiological salt solution and were mixed in equal proportions with a Mengo or a vesicular stomatitis virus suspension representing 100 to 200 tissue-culture doses. The mixtures were incubated as above and were inoculated into tissue-culture tubes. No attempt was made to determine the presence of Bunyamwera virus antibodies in the blood of the surviving mice.

Experimental

Effect of Mengo virus. The 3 preliminary experiments were carried out on small groups of mice subjected to 2½, 5, and 7 months of treatment with benzpyrene (TABLE 1). Virus treatment consisted of from 8 to 13 intra-vaginal applications of viral suspensions, depending on the experiment. The results of examinations of vaginal smears of benzpyrene-treated mice before viral exposure were suggestive of the presence of malignant neoplasms. The early development of carcinoma in mice treated for 2½ months could be attributed to the use of the speculum for visualization of the cervix during each carcinogenic treatment. The ratio of isolation of Mengo virus from vaginal swabbings of benzpyrene-treated and control mice is shown in TABLE 1. It will be observed that, although virus was isolated in the second experiment from vaginal swabbings of nontreated controls, the total number of virus-infected mice in the benzpyrene group was significantly higher than in the control group. No clinical signs of virus infection were noted, and the neoplastic process seemed to progress in the same manner both in the benzpyrene-treated and the untreated animals. Histological confirmations of cytological diagnoses were obtained in every animal.

Effect of vesicular stomatitis virus. The next experiment was undertaken to elucidate several points. The first concerned the possibility of accelerating the process of carcinogenesis by alternate applications of benzpyrene and of Tween 60, which is known to exhibit cocarcinogenic properties.¹² The second involved determining whether the enhancement of susceptibility to viral infection observed in chemically induced tumors was limited to the Mengo virus. The third and last dealt with learning whether the asymptomatic viral infection induced in cancer-bearing mice would lead to the formation of circulating antibodies.

As shown in TABLE 2, one group of mice was treated with benzpyrene alone, another with benzpyrene and Tween 60, and a third with Tween 60 alone; a speculum was used for all treatments. The fourth group was left untreated. At the end of 19 weeks, that is, after slightly more than 4½ months of treatment, there was cytological evidence of malignant neoplasm in the smears of the mice treated with benzpyrene alone; at the end of the same period evidence was inconclusive in the smears of the mice treated

TABLE 2
PRESENCE (+) OF VESICULAR STOMATITIS VIRUS (NEW JERSEY STRAIN) IN VAGINAL
ASPIRATES OF MICE SUBMITTED TO CARCINOGENIC TREATMENT

Carcinogenic treatment	Infectivity of vaginal aspirates (days after virus treatment)		
	3	5	7
Benzpyrene*	+	+	+
Benzpyrene + Tween 60*	0	+	+
Tween 60*	0	0	0
None	0	0	0

The highest ratio of virus isolation for benzpyrene-treated mice was 3/3 (third and fifth day); for benzpyrene- and Tween 60-treated mice, 1/1 (fifth day); for Tween 60-treated mice, 0/3; and for untreated mice, 0/3.

* Treatment for 19 wk.

with benzpyrene and Tween 60, but malignant cells were found in the smears obtained within the next few weeks. The vaginal smears from mice in the last two groups remained negative for malignant neoplasms throughout the entire period of the experiment. It may be concluded, therefore, that partial replacement of benzpyrene by alternate treatment with Tween 60 not only did not lead to acceleration of the neoplastic process, but may have delayed somewhat the appearance of malignancy.

At the end of nineteen weeks the treated and nontreated mice were divided into groups and were infected by the vaginal route with strains of vesicular stomatitis virus and Mengo virus, respectively. As shown in TABLE 2, the New Jersey strain of vesicular stomatitis virus was isolated

TABLE 3
PRESENCE (+) OF VESICULAR STOMATITIS VIRUS (INDIANA STRAIN) IN VAGINAL
ASPIRATES OF MICE SUBMITTED TO CARCINOGENIC TREATMENT

Carcinogenic treatment	Infectivity of vaginal aspirates (days after virus treatment)			
	2	5	9	12
Benzpyrene*	+	+	+	0
Benzpyrene and Tween 60*	+	+	+	0
Tween 60*	0	0		
None	0	0		

The highest ratio of virus isolation for benzpyrene-treated mice was 3/3 (second, fifth, and ninth day); for benzpyrene- and Tween 60-treated mice, 2/2 (second and fifth day); for Tween 60-treated mice, 0/3; and for untreated mice, 0/3.

* Treatment for 19 wk.

TABLE 4
 PRESENCE OF MENO VIRUS IN VAGINAL SWABBING AND OF CIRCULATING ANTIVIRUS
 ANTIBODIES IN THE BLOOD OF MICE INFECTED WITH THIS AGENT

Carcinogenic pretreatment	Ratio of mice with:	
	positive vaginal aspirates	antibodies
Benzpyrene*.....	5/6	3/3
Benzpyrene and Tween 60*.....	5/5	0/2
None.....	2/5	0/2

* Treatment for 19 wk.

only from the vaginal swabbings of the mice pretreated either with benzpyrene or benzpyrene and Tween 60; the virus was still found in vaginal swabbings on the seventh day after its introduction; the titer of the virus indicated active multiplication in the host. Similar results were obtained in mice infected with the Indiana strain of vesicular stomatitis virus, as shown in TABLE 3. Once again the infection of mice subjected to carcinogenic treatment was quite specific, although virus was isolated from the benzpyrene-Tween 60 treatment group at a time when cytological evidence of malignancy was not yet manifest.

The results obtained with the experimental series following infection with Mengo virus are shown in TABLE 4. The ratio of virus-infected vaginal swabbings again was high in mice subjected to carcinogenic treatment. Although 2 of the 5 control mice also showed virus, neither of these 2 showed the presence of Mengo antibodies in blood drawn 4 weeks after virus infection, nor were antibodies noted in the blood of mice treated with benzpyrene and Tween 60. In contrast, antibodies were demonstrated in the sera of mice that became infected with Mengo virus after pretreatment with benzpyrene alone, the only group in which positive evidence of malignancy was found in cytological smears obtained at the time of the first virus application.

Comparison of results obtained with carcinogen and local irritant. Since the application of a carcinogen may act also as a local irritant, in this experiment 1 group of 6 mice was subjected to applications of benzpyrene for 15 weeks (about 4 months) and another group was subjected to a similar number of intravaginal applications of Croton oil; the third group was untreated. The 3 groups were then exposed to viral infection.

The results obtained with the Indiana strain of vesicular stomatitis virus are shown in TABLE 5. The virus was present in vaginal swabbings of 4 out of 6 control mice on the second and fourth day after its introduction. However, a much higher infectivity ratio was obtained in mice pretreated with benzpyrene; the virus was still present in vaginal washings obtained 10 days after introduction of the virus. Mice treated with Croton oil remained refractory to virus infection. The results obtained after the introduction of the New Jersey strain were essentially similar for the benzpyrene- and Croton

TABLE 5
COMPARATIVE EFFECT OF CARCINOGEN AND IRRITANT UPON SUSCEPTIBILITY
OF MICE TO INTRAVAGINAL INFECTION WITH VESICULAR
STOMATITIS VIRUS (INDIANA STRAIN)

Pretreatment	Infectivity of vaginal aspirates (days after virus treatment)			
	2	4	7	10
Benzpyrene*.....	+	+	+	+
Croton oil*.....	0	0	0	0
None.....	+	+	0	0

The highest infectivity ratio in benzpyrene-treated mice was 5/6 on the fourth day.

* Treatment for 15 wk.

oil-treated mice (TABLE 6), but no virus was found in the vaginal swabbings of the nontreated control mice. These results seem to indicate that the enhancement of susceptibility to virus infection is specifically related to the development of neoplasia, and that local applications of strong irritants exercise no effects.

The duration of carcinogenic treatment and susceptibility to viral infection. The objective of the next experiment was to determine the duration of benzpyrene treatment necessary to achieve enhancement of the host's susceptibility to viral infection, and to learn whether the multiplication of virus would inhibit the neoplastic process at any time in the early stage. In contrast to previous experiments, in which small numbers of animals were used, in this experiment 240 mice were divided into 4 equal groups and were pretreated with benzpyrene for 1, 2, 3, and 4 months, respectively. In each

TABLE 6
COMPARATIVE EFFECT OF CARCINOGEN AND IRRITANT UPON SUSCEPTIBILITY
OF MICE TO INTRAVAGINAL INFECTION WITH VESICULAR
STOMATITIS VIRUS (NEW JERSEY STRAIN)

Pretreatment	Infectivity of vaginal aspirates (days after virus treatment)		
	3	6	10
Benzpyrene*.....	+	+	+
Croton oil*.....	0	0	0
None.....	0	0	0

The highest ratio of isolation with benzpyrene-treated mice was 5/6 on the third day. No virus was isolated from the 6 Croton oil-treated and the 6 untreated mice.

* Treatment for 15 wk.

TABLE 7
CORRELATION OF CARCINOGENESIS, SUSCEPTIBILITY TO VIRUS INFECTION,
AND CYTOLOGICAL FINDINGS

Months of benzpyrene treatment	Ratio of virus-infected mice	Mice showing presence of antibodies	Number of mice with smears suggestive of early carcinoma at time of last benzpyrene treatment
1	2/25	1/5	0/20
2	1/25	0/6	0/20
3	3/24	0/5	4/20
4	14/25	11/12	9/20

group only 20 mice were followed up by taking weekly vaginal smears. A week after the last treatment with benzpyrene, applied through the speculum, 25 mice from each group of 60, including 10 of 20 mice followed by taking weekly smears, were exposed to blind intravaginal paintings with vesicular stomatitis virus. The results of this experiment, summarized in TABLE 7, indicate a striking correlation between the early morphologically demonstrable carcinoma and susceptibility to virus infection. After 2 months of benzpyrene treatment, none of the mice showed the presence of neoplastic cells in its vaginal smears, and susceptibility to virus infection was almost nil. The viral infection fared no better in mice subjected to 3 months of carcinogenic treatment, although 4 of 20 mice had neoplastic cells demonstrated in their vaginal smears prior to exposure to the virus. Introduction of the virus into mice pretreated for 4 months with benzpyrene led to a more uniform infection that could be demonstrated either by the appearance of virus in vaginal swabbings or by the presence of antibodies. This seemed to be related to the presence of neoplastic cells in smears of 9 of 20 pretreated animals that were followed up by weekly cytological examinations. That the multiplication of vesicular stomatitis virus did not affect the progress of induced cervical carcinoma at any stage of its development is clearly demonstrated in TABLE 8, which shows the proportion of survivors 7 months after the beginning of benzpyrene applications, both among virus-treated and nonvirus-treated mice.

TABLE 8
PROPORTION OF SURVIVORS 7 MONTHS AFTER BEGINNING OF BENZPYRENE
APPLICATIONS AMONG VIRUS-INFECTED AND NONINFECTED MICE

Months of benzpyrene treatment	Virus-infected group	Noninfected group
1	19/25	30/35
2	18/25	29/35
3	11/25	17/35
4	3/25	6/35

Comment

Results of the above experiments indicate a definite enhancement of the susceptibility of the genital tract of C3H mice to infection with Mengo and vesicular stomatitis viruses in the course of a neoplastic process. Since animals subjected to irritation of the uterine cervix with Croton oil (which does not lead to the formation of neoplastic lesions) remain refractory to viral infection, we may assume that the enhanced susceptibility to certain viruses is dependent upon the neoplastic process itself.

Susceptibility to viruses following carcinogenic applications becomes apparent only when early neoplastic lesions are established. These may be induced slowly by blind intravaginal paintings with benzpyrene, or more rapidly, if such paintings are done under strict visual control and with the use of a speculum. Use of the speculum was originally introduced in the course of this study with the aim of protecting the vagina and vulva from exposure to the carcinogen; it was hoped thereby to induce the formation of tumors exclusively in the uterine cervix. It was observed, however, that this modification resulted primarily in a more rapid formation of cancers, usually involving both the cervix and vagina. For example, after 5 months of benzpyrene treatment, mice in which the speculum was used usually had grossly visible tumors, while the majority of mice treated for the same length of time without the speculum exhibited, at most, only microscopic neoplastic lesions. It is uncertain whether the tumors developed more rapidly merely because there was a more consistent concentration of carcinogen within a small but well-defined area, or whether repeated traumata inflicted upon the cervical tissue by the relatively sharp edges of a metal speculum, played an additional role in accelerating the development of the neoplasms. Whether these factors had any influence, the cytological patterns obtained would suggest that, when the speculum was used, carcinoma occurred earlier in mice treated with benzpyrene alone than in those given alternate treatments of benzpyrene and Tween 60, and that blind paintings of benzpyrene alone resulted in an even later appearance of carcinoma.

Histological sections of the uteri of mice, sacrificed at the time of appearance of the cytological findings considered characteristic of early cervical carcinoma, revealed the presence of small invasive epidermoid carcinomas of the cervix and the vagina. The lesions were usually microscopic; larger tumors were observed later during the neoplastic process. There was an insufficient number of indisputable intraepithelial, that is, *in situ*, carcinomas to attempt a cytological differentiation of such lesions from those in which early invasion was demonstrable. Since cytological evidence of early carcinoma was present in the vaginal smears of mice shown to be susceptible to viral infection and was absent in the smears of animals refractory to such infection, it was concluded that susceptibility to the infection with Mengo and vesicular stomatitis viruses appears when early malignant lesions are already present. We cannot be certain, however, whether or not such susceptibility already exists at the *in situ* stage of the induced cervical carcinoma.

It is of interest to observe that, while virus was occasionally isolated from

the vaginal washings of mice in which cytological evidence of carcinoma was lacking, the demonstration of circulating antiviral antibodies was obtained only from animals in which there was cytological evidence of carcinoma. It should be stressed that the induced viral infection in cancer-bearing mice was totally asymptomatic and did not affect the course of the malignant disease at any stage. At no time did cytological findings indicate oncolysis or include observations of any specific changes attributable to viral infection.

Adaptation of benzpyrene-induced cervical carcinoma to ascites form in C3H mice. Since the results of a previous study⁵ had indicated that, in the case of ascites tumors, multiplication of Mengo virus in cancer cells was accom-

Inoculation of induced cervical carcinoma

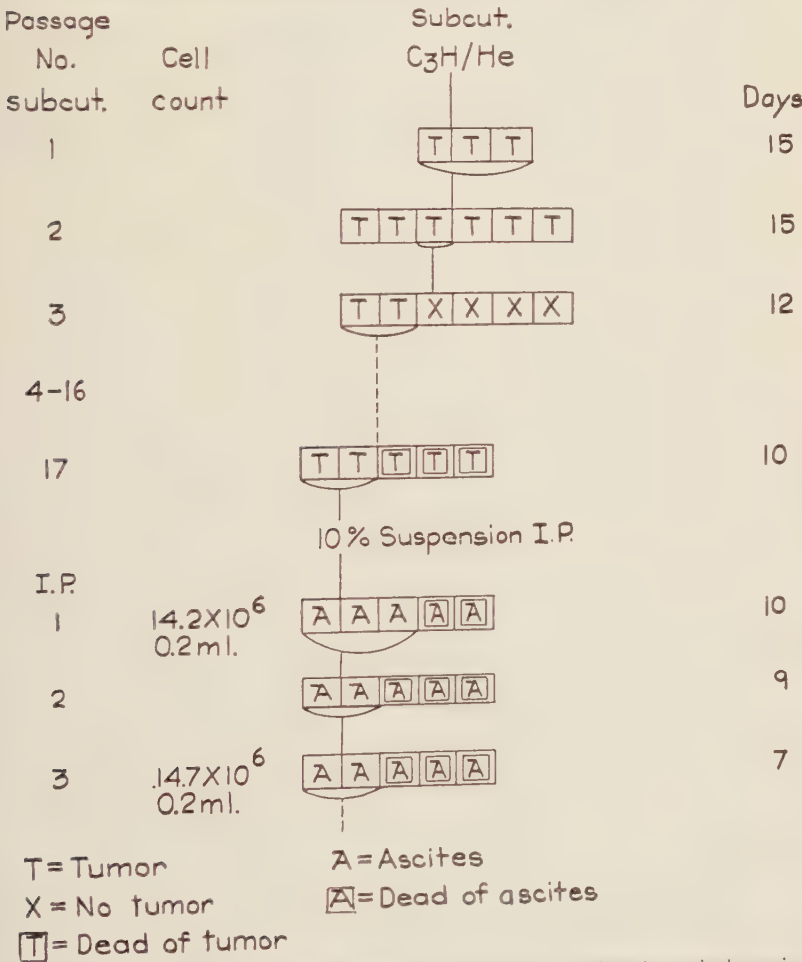


FIGURE 1. History of the transformation of benzpyrene induced cervical carcinoma of the C3H mouse into an ascites tumor through serial subcutaneous and intraperitoneal passages into mice of the same strain.

Suspension of induced cervical carcinoma ICR mice - *in utero*

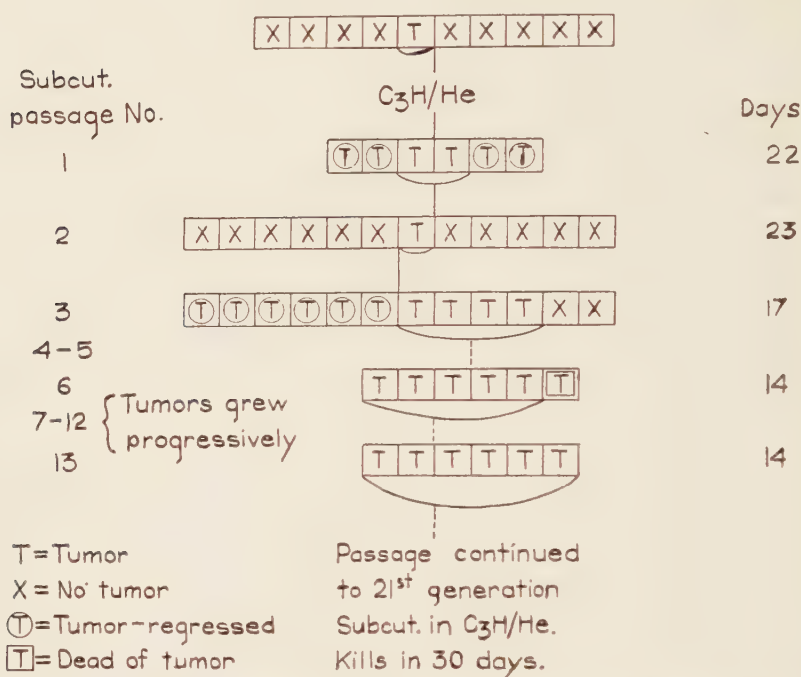


FIGURE 2. History of the adaptation of benzpyrene induced cervical carcinoma of the C3H mouse to subcutaneous growth through the inoculation *in utero* of mice of a homologous ICR strain. This tumor was adapted to subcutaneous growth in C3H mice, but failed to grow progressively in ICR mice.

panied by oncolysis, further investigation was necessary to study the ability of this agent to multiply in induced cervical carcinoma without causing oncolysis. It was thought that, if an ascites line of cervical carcinoma could be established, we should be in a position to re-evaluate the present data.

A cervical tumor was removed from one of the benzpyrene-treated mice, and its fragments were implanted subcutaneously in 3 C3H mice of isologous strain. As shown in FIGURE 1, tumors developed within 15 days after implantation. These 3 mice were sacrificed, and fragments of their tumor tissue were implanted again into 6 C3H mice. Fifteen days later the developing tumor in 1 mouse was removed and was passaged in another group of mice. Following 17 successful subcutaneous transplantations of the tumor, attempts were made (FIGURE 1) to establish an ascites line. The tumors removed from 2 mice on the 10th day after implantation were made into a 10-per cent suspension, and 142 million cells of the pooled fluid were injected intraperitoneally into a group of 5 mice. Ascites developed within 10 days after inoculation, and material containing these tumor cells was

injected intraperitoneally into another group of mice. Fluid obtained from 2 mice of this latter group on the 9th day after inoculation was used for the next passage, in which 3 mice died of ascites tumors. The amount of ascitic fluid was considerable, and freely growing cells were found in the peritoneal cavities of these animals. From that time there has been no difficulty in maintaining the ascites line of the tumor which, at present, is in its 20th passage generation.

Another attempt to adapt the same cervical carcinoma to subcutaneous and intraperitoneal growth was begun by inoculating *in utero* mice of a homologous ICR strain. As shown in FIGURE 2, of 10 fetuses born, 1 developed tumor. The tumor was removed and implanted subcutaneously into adult C3H mice. No tumor grew in the ICR mice, but tumor did develop in 3 C3H mice after an incubation period of 22 days. On the 2nd transfer of the tumor in C3H mice, only 1 animal of the 12 developed tumor. No difficulties were encountered subsequently in establishing a subcutaneous growth of the tumor in C3H mice. FIGURE 2 shows that the incubation period was shortened by 17 to 14 days. At present, the tumor is in its

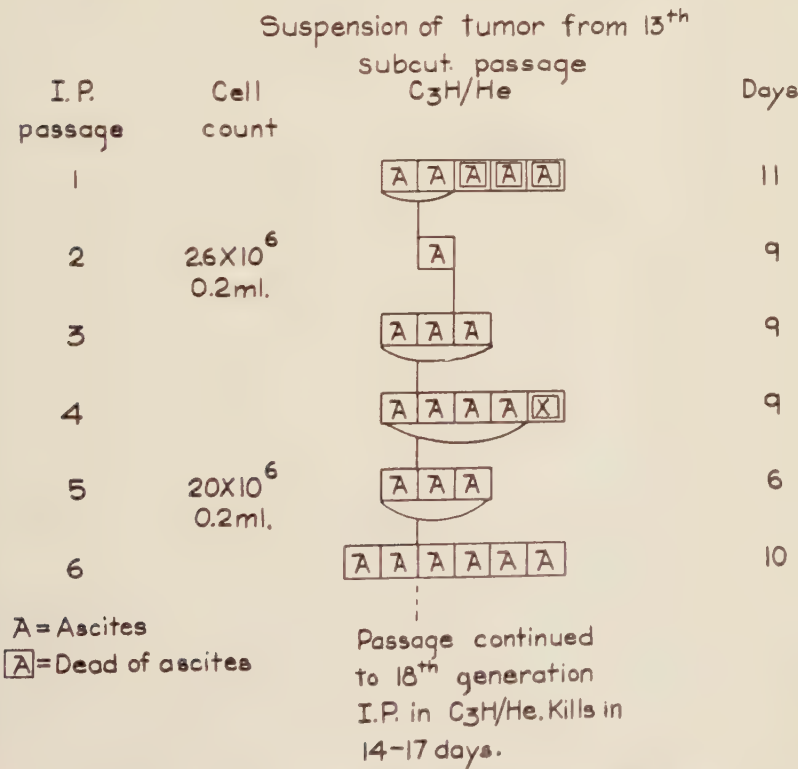


FIGURE 3. History of the transformation of subcutaneous tumors, adapted through the inoculation *in utero* of induced cervical carcinoma into regularly growing ascites tumors.

TABLE 9
COMPARATIVE TITRATION OF BENZPYRENE INDUCED M-70 CERVICAL CARCINOMA
(13 C3H+ ASCITES LINE) IN MOUSE STRAINS, DEMONSTRATING UNALTERED
SPECIFICITY OF THE TUMOR FOR THE ORIGINAL C3H+ HOST

Strain of mice	Mortality ratios of ascites-bearing mice injected with tumor-cell concentrations						
	10 ⁷	10 ⁶	10 ⁵	10 ⁴	10 ³	10 ²	10
C3H/He	5/5	5/5	5/5	5/5	5/5	5/5	2/5
C57/B16	0/5	0/5	0/5	0/5	0/5	0/5	0/5
DBA/2	0/5	0/5	0/5	0/5	0/5	0/5	0/5
AJAX	2/5	0/5	0/5	0/5	0/5	0/5	0/5
ICR	0/5	0/5	0/5	0/5	0/5	0/5	0/5
PRI	2/5	0/5	0/5	0/5	0/5	0/5	0/5

21st generation, and causes the death of animals within 30 days after implantation.

The tumor originating from the 13th subcutaneous passage of this line was made into 10-per cent suspension and, as shown in FIGURE 3, was injected intraperitoneally into a group of 5 C3H mice. All animals developed ascites, and 3 died. An inoculation of 2 million cells, drawn from the 2 living mice, was used for the next passage. This line has been maintained in ascites form without difficulty. At present, the tumor is in its 18th generation, and is lethal to C3H mice— an inoculum of 20 million cells causes death within 14 to 17 days after inoculation.

The remarkable specificity of this ascites line of cervical carcinoma in C3H HE mice is shown in TABLE 9. An inoculum consisting of 10 cells is sufficient to kill 2 of 5 animals of this strain. In marked contrast, none of the other strains of mice tested is susceptible to growth of the tumor. Only 2 mice of the Ajax and the PRI strain, respectively, died after intraperitoneal inoculation with 10 million tumor cells.

Since the original cervical carcinoma supported the growth of vesicular stomatitis viruses, while the neoplastic process did not seem to be arrested by viral infection, we thought it worthwhile to verify the susceptibility of the ascites line of the tumor to virus infection. Groups of C3H mice inoculated with the 13th intraperitoneal passage of tumor were injected 5 days later with the 4 viral agents listed in TABLE 10. The mice were tapped daily for evidence of cytological destruction; 5 days after the viral injection, enough ascites fluid was obtained to perform titration and bioassays. The results indicated that the 2 strains of vesicular stomatitis virus multiplied in the tumor cells without causing oncolysis. Multiplication of Bunyamwera virus also occurred without affecting the development of the tumor. There was some evidence of destruction of tumor cells after inoculation with Mengo virus, but no bioassay was feasible, because all test mice died from virus infection.

TABLE 10
EFFECT OF VIRUS INFECTION ON ASCITES TUMOR THAT ORIGINATED
FROM BENZPYRENE-INDUCED CERVICAL NEOPLASM

Virus	LD ₅₀ inoc. (log)	Oncolysis	LD ₅₀ titer of ascitic fluid (log)	Bioassay- tumor ratio in mice
Vesicular stomatitis (Ind.).....	2.0	None	7.0	5/5
Vesicular stomatitis (N. J.).....	2.0	None	4.5	5/5
Bunyamwera.....	3.0	None	3.5	5/5
Mengo.....	3.0	±	4.5	0/0*
None.....	—	—	—	5/5

* All mice died from virus infection.

Summary

By means of vaginal paintings with benzpyrene, a chemically induced, strain-specific tumor developed in an inbred strain of mice. This cervical carcinoma was shown to support the growth of Mengo and vesicular stomatitis (New Jersey and Indiana strains) viruses. The course of infection was asymptomatic and could be determined only by isolation of virus from vaginal swabbings or through the development of specific circulating antibodies. There was some evidence that enhancement of the susceptibility of mouse hosts to viral infection had already occurred at the time when cytological evidence of malignancy was not yet fully established. In spite of multiplication of viral agents in the neoplastic cells, there was no evidence of oncolytic action. Adaptation of the chemically induced, cervical carcinoma to growth in the ascites form enabled more quantitative studies with the tumor, which led to a confirmation of the results of the original experiments, namely, that the same viral agents multiply in the ascites tumor without exercising oncolytic action.

Acknowledgments

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LYMPHOCYTIC CHORIOMENINGITIS VIRUS AS RELATED TO CHEMOTHERAPY STUDIES AND TO TUMOR INDUCTION IN MICE

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The virus of lymphocytic choriomeningitis (LCM) in mouse neoplasms merits consideration in relation to (1) experimental chemotherapy of tumors, and (2) tumor induction.

LCM Virus and Chemotherapy Studies

In 1956 Stewart and Haas¹ recovered and identified LCM virus in 2 sublines of L 1210 leukemia‡. One subline designated AMD, FX, reported as A-Methopterin dependent, was obtained from A. Goldin, and the other, subline C, G 153, an ascites tumor carried with 8-azaguanine to the 98th transfer, was obtained from L. W. Law§. Since optimal growth of both sublines occurred in the presence of drugs, the question arose as to whether drug action was in any way conditioned by the presence of the virus.

Haas and Stewart,⁴ and Haas *et al.*,⁵ have reported on the sparing effect of A-Methopterin and, to a lesser degree, of 8-azaguanine on mice inoculated with otherwise uniformly lethal doses of LCM. The drugs did not prevent virus survival and replication, but they did prevent death of a large proportion of the mice inoculated with the virus.

Humphreys *et al.*⁶ reported on a contaminant in the same AMD, FX A-Methopterin-dependent subline of leukemia L 1210 referred to above; this agent retarded growth of the tumor if A-Methopterin was withheld. The contaminant was identified as LCM virus.¹ The tumor, ordinarily growing optimally in the presence of A-Methopterin, developed equally well in mice immunized against the contaminating agent, even though A-Methopterin was withheld. The "contaminant-free" leukemia (tumor passed through immune mice) was relatively resistant to A-Methopterin.

These reported findings are of interest to experimental chemotherapy of mouse tumors, for mice are known to harbor latent viruses, one of which is LCM. It is conceivable that a latent virus in tumor cells, or elsewhere in the host, could be activated by the drug under study. The presence of an active virus in a tumor could make it difficult to interpret the results of chemotherapy studies.

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‡ This leukemia arose in a strain-DBA/2₁₂₉ mouse that had been treated with methylcholanthrene.² In 1951, Law and Dunn³ reported on the effect of a filterable self-propagating contaminant on a transplantable subline of this leukemia. The contaminant was not identified.

§ Both of the National Cancer Institute.

LCM Virus and Tumor Induction

In 1941, Traub⁷ reported a higher incidence of leukemia in a mouse colony infected with LCM virus than in a subline of this colony that was free of the virus. Some of the leukemias from which Stewart and Haas¹ recovered LCM yielded cell-free extracts with high tumor-inciting properties.

These findings suggested the desirability of further studies on the possible role of LCM virus in tumor induction in mice. In the work reported here, LCM could not be implicated as the agent responsible for the neoplasms produced. The reasons for this are as follows:

(1) As reported earlier, cell-free extracts of leukemia No. 60, an AK-n leukemia carried by transplantation in AKR mice, have repeatedly induced tumors. LCM virus was recovered from this leukemia, but cell-free extracts of a subline freed of LCM virus by 20 passages through mice immunized against the virus were still capable of inciting tumors.

(2) Normal C3H embryonic thymic tissue mixed with cell-free extracts of leukemia No. 60 and implanted subcutaneously into 4- to 6-week-old irradiated strain C3H He male mice developed into lymphocytic neoplasms in 12.8 per cent of the mice. Genetically, the lymphomas were C3H tumors. Normal C3H embryonic thymic tissue contaminated with LCM virus and implanted subcutaneously into similar mice failed to develop into neoplasms.

(3) Four- to 6-week-old Swiss mice given subcutaneous injections of tumor-inducing material, in which LCM virus could not be demonstrated, later succumbed to an intracerebral challenge with LCM.

Materials and Methods

Tumors. Two tumors were used in this study:

(1) AK-n leukemia No. 60, which is carried in transplant in AKR mice. At the time of its 72nd transplant generation a subline was started in AKR mice that had been immunized¹ against LCM virus. Transplants were made every 8 to 9 days in the immune mice for 20 passages. After these passages, LCM virus could no longer be recovered by the usual methods of testing.¹

(2) Parotid gland tumor No. 3469, which arose in a (C3Hf \times AKR) F_1 hybrid mouse that had been inoculated with a tissue-culture preparation from a spontaneous AKR leukemia (24424).² (Tissue-culture preparations of this tumor were found to induce neoplasms.⁸)

Tissue culture. Tissue cultures were prepared as previously described.² Since further observations were made by using the same technique, we shall present a short review of the preliminary work. Tissue cultures prepared from adult monkey kidney and from chick chorioallantoic membrane were inoculated with homogenates of cell suspensions of spontaneous AKR leukemias, primary parotid gland tumors, and transplanted C3H and AK-n leukemias. These were incubated at 36° C. At weekly intervals the fluids were removed from the cultures and new culture medium was added. Transfers of fluid to new tissue cultures were usually made at the same time. For

TABLE 1
TUMOR INDUCTION IN HYBRID MICE INOCULATED WITH FLUIDS FROM TISSUE
CULTURES THAT RECEIVED CELL-FREE EXTRACT OF LEUKEMIA NO. 60
FREED OF LCM VIRUS (5-MONTH OBSERVATION PERIOD)*

Tissue culture	Tissue cultures inoculated with cell-free leukemia extract						Uninoculated tissue culture		
	Culture passage	Weeks incubation	Mice inoculated	Mice surviving	No. developed neoplasms	Age when tumors appeared (months)	Mice inoculated	Mice surviving	Mice developed neoplasms
Monkey kidney	1	2	4	4	3	3-5	4	4	0
	2	1	4	2	2	4	4	4	0
	3	2	3	2	2	3	3	3	0
Chick chorioallantoic membrane	1	2	3	3	2	4	4	4	0
	2	2	6	6	5	3-5	5	4	0

* The mice developing neoplasms all had parotid gland tumors and renal cortical lesions; the others had one or more of the other epithelial tumors described. None developed leukemia.

controls, an equal number of uninoculated monkey kidney and chick chorioallantoic membrane tissue cultures, from the same cells used, were maintained with every experiment, and fluids from these controls were passed to new cultures whenever passages of the test materials were made. Fluids from the controls and from the inoculated tissue cultures were injected subcutaneously into newborn mice of different strains to determine tumor induction, and were injected intracerebrally into 4-week-old Swiss mice to test for LCM or other viruses pathogenic for mice.

The neoplasms and lesions produced by the culture preparations were generally of types not found to occur spontaneously in mice. All were tumors and lesions of epithelial cells.

In the present study, monkey kidney and chick chorioallantoic membrane tissue cultures were inoculated with the following: (1) a cell-free extract* prepared from LCM-free tumor of leukemia No. 60; (2) a homogenate prepared from primary parotid gland tumor No. 3469; and (3) a 10^{-2} dilution of known LCM virus in a mouse brain suspension. These were carried in the cultures as described.⁸ After 1- and 2-week incubation at 36° C, the supernatant fluids from the cultures were tested for: (1) tumor-inciting properties, by inoculating subcutaneously into newborn (C3Hf × AKR)F₁ mice, and (2) the presence of LCM virus, by inoculating intracerebrally into

* The extracts were prepared as described previously.⁹ The presence of cells in the extracts was ruled out by genetic studies of the tumors induced.

4-week-old Swiss mice. Cross immunization against LCM virus was done with eighth-passage, tumor tissue-culture material.

Immunization. Adopting the procedures used for immunizing against LCM virus,¹ groups of 5 each of 4-week-old Swiss mice were inoculated subcutaneously with 0.25 ml. of tissue culture fluids from: (1) eighth passage from cultures inoculated with cell-free extract from leukemia No. 60 (subline free of LCM virus); (2) eighth passage from cultures that had been inoculated with cells from parotid gland tumor No. 3469; and (3) fifth passage from cultures of LCM virus. Ten days later they were challenged intracerebrally with 0.03 cc. of a 10^{-3} dilution of LCM virus in a mouse brain suspension.

Swiss mice that had survived intracerebral inoculation two weeks previously with tissue-culture preparations found to induce neoplasms were also challenged with LCM virus.

Mice. The strain AKR mice were obtained from the Jackson Memorial Laboratory, Bar Harbor, Me. All others were from stocks of the National Institutes of Health, Bethesda, Md. All newborn mice and embryos were from mice bred in the laboratory. Newborn mice were used before they were 12 hours old. All mice were housed in plastic cages and were constantly supplied with Purina laboratory chow and tap water. Greens were supplied once a week.

Thymic implants. Cell-free extracts were prepared from leukemia No. 60 (the subline that carried LCM virus) by the same method described earlier,⁹ except that the homogenizers and the Ringer's lactate solution were cooled to 4° C. before being used. Fresh extracts were made for each experiment. The method used for the subcutaneous thymic implants was similar to that described earlier, when the implants were made into the anterior chamber of the eye of the host.⁹ A review of the method, with the modifications, follows:

Sixteen- to 18-day-old C3H embryos were removed from pregnant, apparently normal, strain C3H He females. The thymus was removed aseptically from each embryo and was divided into halves; one lobe was placed in each of 2 tissue dishes containing just enough Ringer's lactate solution to prevent drying or maceration of the tissues. In order to use fresh tissue, not more than 10 to 12 thymi were removed from embryos for each experiment. The thymic lobes from 1 set were injured by a slight pricking with a pair of sharp scissors; sufficient Ringer's lactate solution was added to cover the tissue completely. These lobes were used for inoculating the control mice. Those from the other set were removed and placed in a tissue dish containing the No. 60 leukemia cell-free extract and, while immersed in the extract, were pricked in like manner. In order to avoid all possible cross contamination from handling, this second set of thymic tissue was not added directly to the leukemic extract when first removed from the embryos. The tissues were allowed to stand in the respective solutions for 15 to 20 min. and, by means of trocars, were then inoculated subcutaneously into 4- to 6-week-old male litter mates of strain C3H He mice that had been treated with 250 r* total-

* The physical factors for irradiation were: total-body irradiation 1 tube; 250 KVP, 30 μ ; beam filtration, 1.0 mm. Cu + 1.0 Al target-mouse distance for tube, 50 cm.; dose rate 75 r per minute.



FIGURE 1. Bilateral salivary gland tumors and an epithelioid thymic tumor in a female mouse. The thymic tumor fills the thoracic cavity almost completely. $\times 3$.

body irradiation 1 to 2 hours earlier. The implants, which consisted in each instance of an entire thymic lobe, were made in the right axillary region.

LCM-contaminated embryonic thymic tissues prepared by the same method were implanted subcutaneously into irradiated C3H/Hen male mice.

Results

It was reported earlier⁸ that LCM virus was recovered from 2 of 28 tumor tissue-culture preparations. Eight of these produced neoplasms in a high percentage of mice inoculated when newborn; another, which was a culture of leukemia No. 60 that had contained LCM virus, killed all newborn mice inoculated, although cell-free extracts of this leukemia were shown to produce neoplasms in many of the similarly inoculated mice.⁸ In the present study it has been found that a subline of leukemia No. 60 freed of LCM virus by passage through LCM immune mice still has an agent capable of inducing neoplasms. Fluids from monkey kidney and chick chorioallantoic membrane tissue cultures inoculated with cell-free extracts of leukemia No. 60

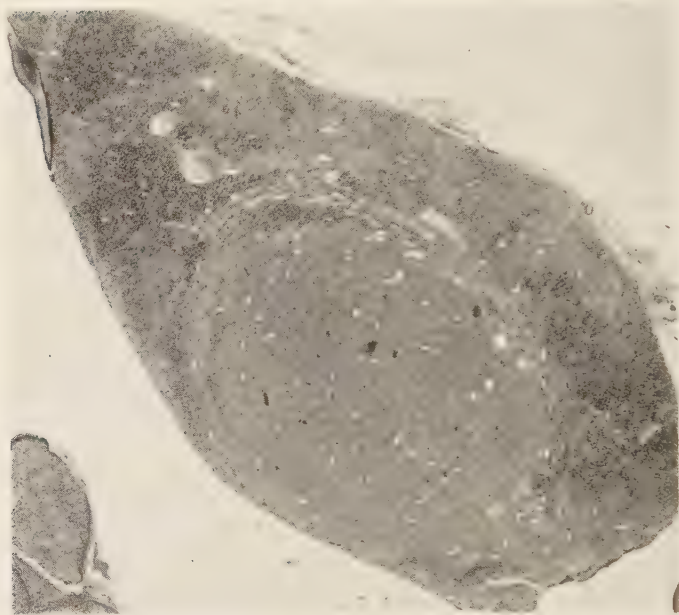


FIGURE 2. Early epithelioid thymic tumor, showing its medullary origin. $\times 27$.

that had undergone passage through LCM-immune mice induced tumors in many of the (C3Hf \times AKR) F_1 hybrid mice inoculated shortly after birth (TABLE 1). Inoculated intracerebrally into 4-week-old Swiss mice, these same fluids produced no signs of LCM infection. The neoplasms induced were epithelial; as described before,⁸ all mice with neoplasms had parotid gland tumors and a proliferative renal cortical lesion of the convoluted tubules. Many also had tumors of the submaxillary and sublingual glands and an epithelioid thymic tumor. Adrenal medullary tumors and early mammary tumors were also induced. The renal cortical lesions and thymic tumors are of special interest, as they had not previously been described.⁸ FIGURES 1 to 5 show tumors and lesions in (C3Hf \times AKR) F_1 hybrid mice that received fluids from tissue cultures inoculated with cell-free extracts of leukemia No. 60 (subline free of LCM). The sections are stained with hematoxylin-eosin.

Eighty C3H mice received subcutaneous implants of C3H embryonic thymus exposed to cell-free extracts of leukemia No. 60. Ten of the 80 mice died early*, and 9 of those remaining developed lymphomas within a 14-month observation period, 4 of these within 90 days after receiving the implant. In 7 of the leukemic mice the tumor arose at the site of the implant (FIGURES 6 and 7); in the others the lymphoma had become generalized. At the time the animals were sacrificed it was found that the tumors

* The subline of leukemia No. 60 carrying LCM virus was used; the deaths may be attributed to LCM virus, as they died within 2 weeks after inoculation of the implants.

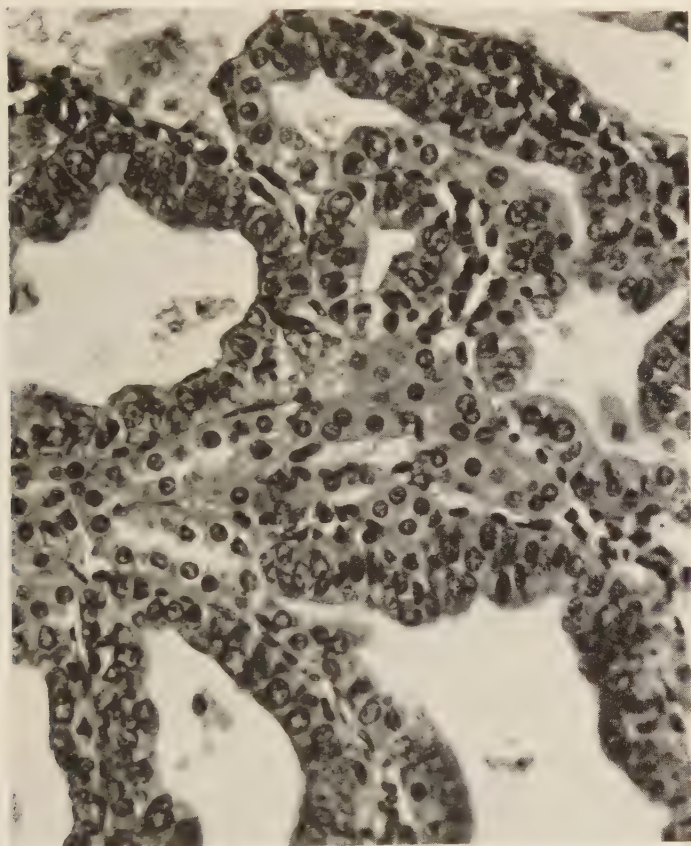


FIGURE 3. Cortical lesions of kidney tubules. $\times 343$.

in 2 had involved only the homolateral axillary nodes. Genetic studies with 7 of the 9 leukemias (2 not done) carried out by subcutaneous transplantation of the lymphoma into strains C3H and AKR mice showed that the tumor cells were the descendants of the cells of the thymic implants and were not derived from any cells that may have been in the extracts of leukemia No. 60. In the same observation period 80 control mice that received the thymic implants that had been immersed only in Ringer's lactate solution remained free of lymphomas. Of a third group of 24 mice that received implants of thymus contaminated with LCM virus, 3 died early, while the others have remained free of lymphomas throughout a 9-month observation period. Twenty-four control mice, similar to the controls described above, also remained free of lymphomas (TABLE 2).

Groups of 5 each of Swiss mice 4 weeks old were inoculated subcutaneously with fluids from eighth-passage tissue cultures of cell-free extracts of leukemia No. 60 (subline free of LCM) and of parotid gland tumor No. 3469, both of which were shown to be active in inducing neoplasms; none survived

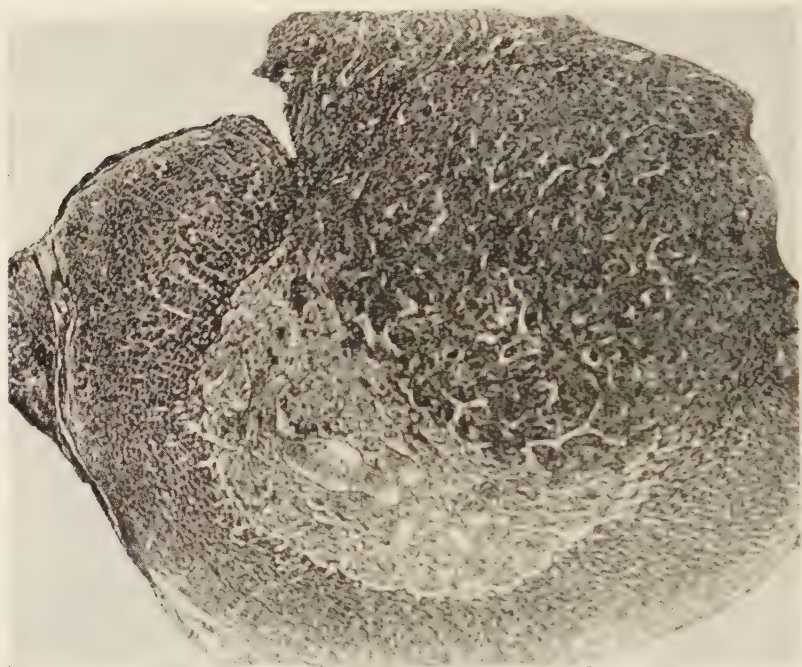


FIGURE 4. Adrenal medullary tumor breaking through the cortex. $\times 52$.

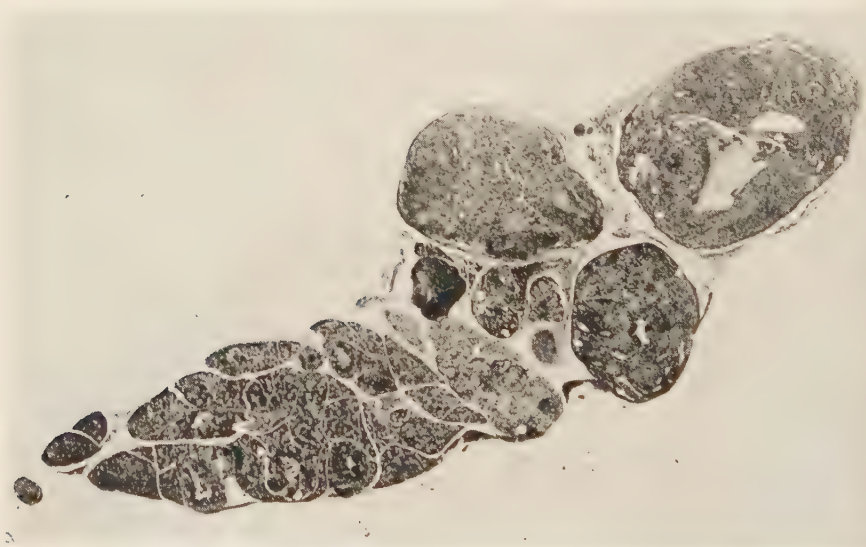


FIGURE 5. Pleomorphic tumors of the submaxillary, sublingual, and parotid glands. $\times 15$.



FIGURE 6. Four-month-old strain C3H male with lymphoma originating in thymic implant that had been exposed to a cell-free extract of leukemia No. 60.

subsequent intracerebral challenge with LCM 2 weeks later. Twenty Swiss mice inoculated intracerebrally with tissue-culture preparations that had been shown to induce tumors when inoculated into newborn hybrid mice also failed to survive challenge with LCM virus. Mice inoculated subcutaneously with fifth-passage tissue-culture fluids from an LCM culture survived LCM challenge 2 weeks later.

Discussion

These studies have revealed no evidence that the tumor-inducing agent in the tissue-culture preparations or in the cell-free leukemia extracts is an LCM virus. While the agent carried in the tissue cultures has characteristics attributable to a virus, no relationship to LCM has been demonstrated.

The agent in the tissue-culture fluids and in the cell-free leukemia extracts that produced parotid gland tumors and other epithelial neoplasms and

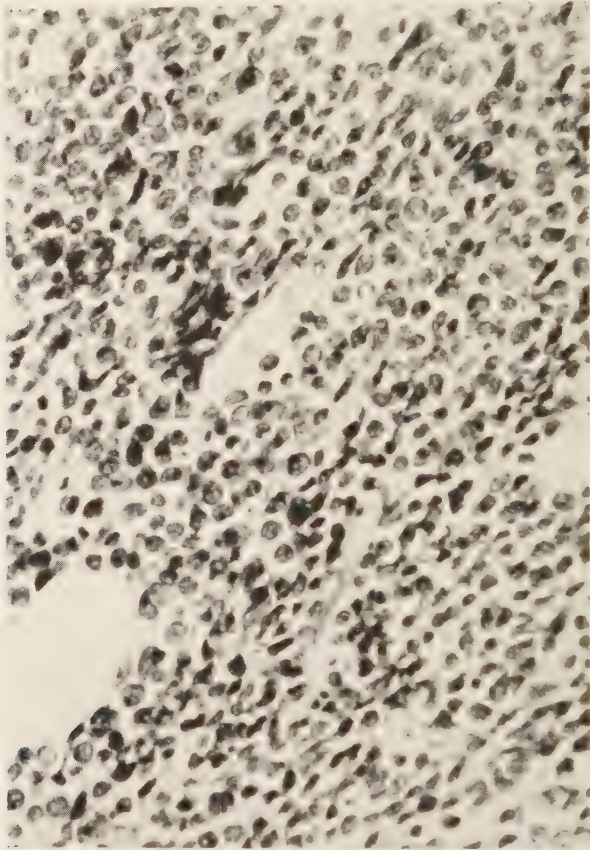


FIGURE 7. Same lymphoma shown in FIGURE 6. $\times 515$. Hematoxylin-eosin stain.

lesions may be different from the substance that produced the leukemia in the embryonic thymic implants, since none of the tissue-culture preparations produced leukemia. This may have been, however, because of the involvement of the thymus in the epithelioid tumor described earlier.⁸ In 1935 Murphy¹⁰ suggested the analogy between pneumococcus-type transformation and the transformation of nonmalignant cells by a cell-free agent. This suggested interpretation assumed that the product of a characteristic malignant cell acts on an undifferentiated cell, causing it to assume the peculiar characters of the specialized one; in this new form the cell produces more of the agent capable of inducing the transformation of other cells. Furthermore, the new cell must pass all of its properties to its descendants. Recently, Furth¹¹ and his associates, working with mouse leukemias, obtained results that support this concept. Our results with the embryonic thymus immersed in the cell-free leukemia extracts, which developed into lymphomas after implanting into mice, indicate that a similar transformation or transduction may have occurred.

TABLE 2
LYMPHOMAS IN EMBRYONIC C3H THYMI IMPLANTED SUBCUTANEOUSLY
IN STRAIN-C3H MICE

Implant	Mice inoculated	Mice surviving	Mice developing lymphomas	Observation period (months)
Thymus + Ringer's lactate solution.....	80	80	0	14
Thymus + No. 60 leukemia extract.....	80	70	9	14
Thymus + Ringer's lactate solution.....	24	24	0	9
Thymus + LCM virus.....	24	21	0	9

Acknowledgment

We gratefully acknowledge the assistance of Alice M. Gochenour and George Grubbs during the course of these investigations.

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PRELIMINARY STUDIES ON THE DEVELOPMENT OF NEOPLASIA IN THE SKIN OF MICE PAINTED WITH METHYLCHOLANTHRENE AND INJECTED WITH CORTISONE AND VACCINE VIRUS*

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The experiments to be described here are part of a general study carried out in our laboratory with the object of discovering whether ordinary viruses (that is, viruses that, under ordinary circumstances, induce immediate, more or less inflammatory lesions that end in necrosis) can start or cause neoplastic and malignant lesions under other circumstances. The concepts upon which this line of research is based can be discussed here only in a broad, general way, and may be summarized as follows:

(1) Concerning such factors as composition, morphology, antigenicity, and epidemiology, one finds the same properties, in the same degree of variability, in viruses that induce neoplasia and cancer (in such animals as birds and mice) as in ordinary viruses. Even at the risk of expressing a platitude, the basic difference between both groups of viruses is that the gross, clinical lesions, *as usually perceived by the observer*, result either in cell stimulation or in cell destruction. This difference reduces itself to various degrees of compatibility between the virus and the cell, ranging from (a) temporary cell stimulation, as in pox viruses, to (b) longer-lasting cell stimulation, as in rabbit fibroma and (c) indefinite cell stimulation, as in sheep pulmonary adenomatosis or avian tumors.

(2) The cell-stimulating or cell-destroying and inflammatory effects of the *same virus* are conditioned, in whole or in degree, by (a) the variation of the virus, as in different strains of pox and fibroma viruses; (b) the age of the host, as in sheep pox and avian tumors; and (c) the phase of the disease; that is, the stimulation of the cell preceding its necrosis, as in pox viruses, or following it, as in sheep pulmonary adenomatosis and in influenza.

The problem of the compatibility of the virus and the cell, however, reduces itself ultimately to the common and fundamental factor^{1,2} of the degree of severity of the infection relative to the degree of virulence of the virus. Generally speaking, acute inflammation and cell necrosis occur in the highly susceptible host attacked by a highly virulent virus whereas, if a balance of power is established, either by a weakening of the infectious agent or by a critical degree of strengthening of the host defenses, cell proliferation will ensue.

* The part of this study dealing with the role of the ground substance of the mesenchyme in natural resistance to infection was supported by Research Grant USPHS A-561 (C8) from the National Institute of Arthritis and Metabolic Diseases, Public Health Service, Bethesda, Md. The remainder of the study was supported by grants from the Jane Coffin Childs Memorial Fund for Medical Research, New Haven, Conn.; the National Cancer Institute, Public Health Service, Bethesda, Md.; and The American Cancer Society, New York, N. Y.



FIGURE 1. Effect of hormone treatment on the dermal ground substance of female Swiss mice. The mouse at the right, showing thickening of the skin, was treated with 100 γ of estradiol benzoate; that of the middle, showing thinning of the skin, was treated with 5 mg. of cortisone; and that of the left was an untreated control.

Pox viruses were chosen for our work because of the broad host range of the virus in the case of vaccinia, and because the duality of effects on cells, dependent on most or all of the factors listed above, is especially manifest with these viruses.

Elsewhere³ my associates and I have reported results with fowl pox virus comparable in several ways to those to be described here. While studies on this virus have been continued, and will be reported in the future, the model system of vaccinia in mice has proved far more convenient for experimentation.

The present experiments indicate that infection by vaccinia [or, more cautiously, that changes occurring in the lesions induced by this virus in mice, prepared with a special combination of methylcholanthrene (MC) and cortisone] result in the *start* of lasting neoplasia and malignancy. We are not concerned at present with the *perpetuation* of the neoplasia as a direct result of the virus infection, although such a possibility will be discussed briefly.

The experiments evolved from our laboratory studies dealing with the general problem of the role played by the mesenchyme and its ground substance in natural resistance against infection and cancer.⁴⁻⁶ Since it is known that some hormonal effects result in pronounced changes of this ground substance, we looked for a model system in which both an increase and a decrease of the ground substance could be induced easily and regularly in the same animal. Such a system was found in young noninbred female Swiss mice.

When these animals were injected subcutaneously with 100 γ of estradiol benzoate (EB), there was a rapid and marked production of dermal ground substance, selectively occurring in the flanks and the back of the mouse. The phenomenon was grossly manifested by a thickening of the skin, sometimes detectable 1 day after inoculation, that attained its maximum at the fourth or fifth day, and persisted for about 2 weeks (FIGURE 1). When stained with toluidine blue, the thickened skin gave a strong metachromatic reaction, and it yielded extremely viscid extracts; both the metachromasia and the viscosity disappeared promptly upon the addition of hyaluronidase.

On the other hand, opposite phenomena were induced by cortisone. When this hormone was injected subcutaneously into normal mice in 5 successive daily doses of 1 mg. each, a thinning of the skin of the flanks and the back was rapidly induced (FIGURE 1), and the metachromatic material, which normally exists in appreciable amounts in these regions, disappeared entirely. The same phenomena were observed when cortisone was injected into mice in which large amounts of ground substance had been induced by previous EB treatment.

As summarized elsewhere,⁶ several studies have been carried out in Swiss mice thus treated in order to learn about the physiology of the ground substance (as a problem of intrinsic interest) and about the behavior of several infectious agents (and also cancer cells) when injected intradermally in different regions of the skin, that is, in the ground substance variously altered by previous treatment with hormones.

One of the infectious agents was vaccinia, a very virulent strain of Levaditi neurovirus kept by passages through rabbit testes. Normal female Swiss mice, the noninbred strain from Carworth Farms, New City, N. Y., proved to be quite resistant to this virus. An intradermal inoculation of 0.1 cc. of a 1:10 extract of the infected rabbit-testicle tissue generally would induce, 5 or 6 days after inoculation, lesions measuring only a few mm. in diameter. This natural resistance against the virus was further increased by treatment with a single dose of 100 γ of EB; when the virus was injected intradermally 5 days after the hormone it induced either no gross lesions or lesions measuring 1 mm. or less in diameter. Cortisone, however, had an entirely opposite effect; when the virus was injected intradermally between the third and fourth daily injection, at a total dose of 5 mg. of the hormone, it regularly induced, by the fifth to the sixth day, severe lesions that often measured 1 cm. or more in diameter. These lesions healed in 2 to 3 weeks, although leaving large and conspicuous scars that persisted for a considerable time.

These pronounced and opposite effects which estrone and cortisone, respectively, produced on the *immediate*, acute vaccinia infection in a host so susceptible to naturally occurring and experimentally induced cancer, prompted us to study again the possibility that, as a *late* effect of the virus infection, neoplasia may develop in specially prepared hosts.* For this purpose vaccine virus was injected into mice having received previously a number of paintings of MC, alone and in combination with either EB or cortisone, to test whether neoplasia would evolve in a way different from that induced by the MC and/or the hormones, in the absence of virus infection.

Obviously, as in past experiments with fowl pox in chickens and with vaccinia in rabbits, such planning of the experiments was based on another tentative assumption, or rather on a further elaboration of our main hypothesis; that is, that carcinogens would be instrumental in the hypothetical induc-

* In the past, the same problem, was extensively studied in rabbits prepared by skin paintings with MC and later injected intravenously and intradermally with vaccinia. As a general conclusion, it can be stated that the virus infection did not appreciably modify the usual events ending in dermal carcinogenesis.

tion of neoplasia by "ordinary" viruses, as they are known to be in some cases of induction of cancer by "neoplastic" viruses—as is the case of MC in the papilloma-carcinoma sequence in the rabbit, and in the case of estrone in the breast cancer of mice.

Accordingly, the Swiss mice were painted, on both flanks, 10 times in 12 days, with a 1 per cent solution of MC in benzol; 3 days after the last painting they were injected, some with 100 γ of EB in a single dose, and some with 5 mg. of cortisone in 5 daily doses. Vaccine virus—0.1 cc. of a 1:10 suspension of infected rabbit testicle—was injected intradermally on the fourth day after the administration of EB and between the third and fourth injection of cortisone. The virus was also injected into mice prepared only with EB, cortisone, and MC, respectively; into normal mice; and into other groups of mice painted with benzol, with and without hormone treatment. Other control groups included mice having received the same treatment, as described above, but not injected with the virus.

In this experiment, treatment with MC did not change (at least in any pronounced degree) the course of the dermal virus infection, either in the mice not treated with hormones that showed the same natural resistance to the infection (FIGURE 2a); in the mice treated with estrone, that showed the same increase of the natural resistance (FIGURE 2b); or in those treated with cortisone, that showed the same enhancement of the infection (FIGURE 2c). Neither did the treatment with MC modify essentially the histological character of these lesions, namely, temporary epithelial stimulation followed by inflammation and necrosis. Benzol treatment likewise had no effect on the virus infection.

The presence of the virus in the lesions from all the groups of mice was checked, in one experiment, by titrating extracts of the lesions in the rabbit skin at the fourth, seventh, eleventh, and eighteenth day after infection. The persistence and the titer of the virus appeared to agree with the persistence and severity of the lesions observed in the differently prepared hosts. Thus, the results were as follows: in the mice treated with MC and EB the virus persisted until the fourth day with a titer of 10^{-2} ; in the mice prepared with MC alone the virus persisted until the seventh day with a titer of 10^{-3} ; and in the mice treated with MC and cortisone the virus persisted until at least the eleventh day with a titer* of 10^{-6} . These preliminary results, therefore, indicate that, in the skin of mice treated with MC and cortisone, the virus finds a better ground for its multiplication than it does in the mice treated otherwise.

In view of these findings, and also for reasons to be given later, we attempted to maintain the virus by skin passages not only through normal mice, but also through differently treated mice. At the present stage of the experiments, it can be said only that, in the mice treated with both MC and cortisone, characteristic skin lesions have been induced regularly in eight successive passages. These lesions are less inflammatory and more prolifera-

* The data on the virus titer may be subject to variation depending on whether only the strict necrotic lesion or larger pieces of skin are extracted. This point is now being studied.

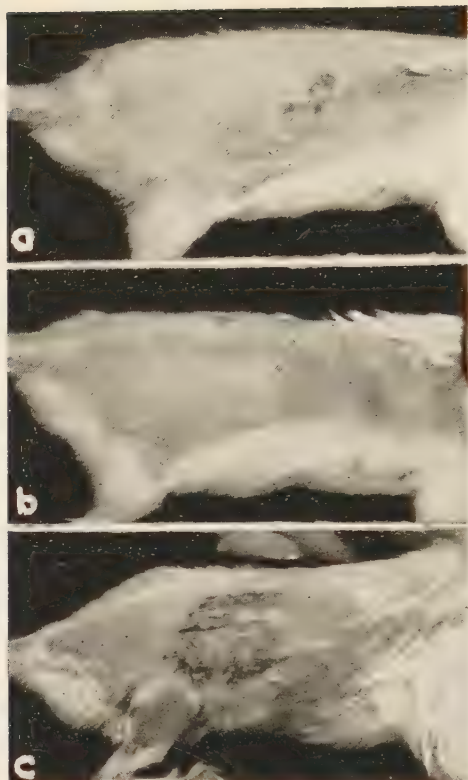


FIGURE 2. Lesions induced by vaccine virus in mice painted with methylcholanthrene and then injected with estradiol benzoate (2b), cortisone (2c), or not injected (2a). The results are 6 days after intradermal injection of the virus.

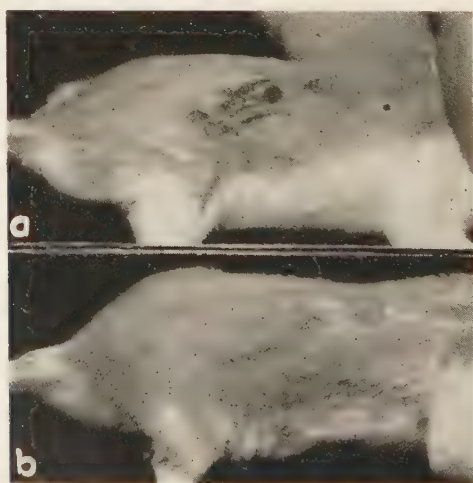


FIGURE 3. Acute vaccine lesion 7 days (3a) after virus injection in a mouse prepared by methylcholanthrene and cortisone, and the scar left after 26 days (3b).

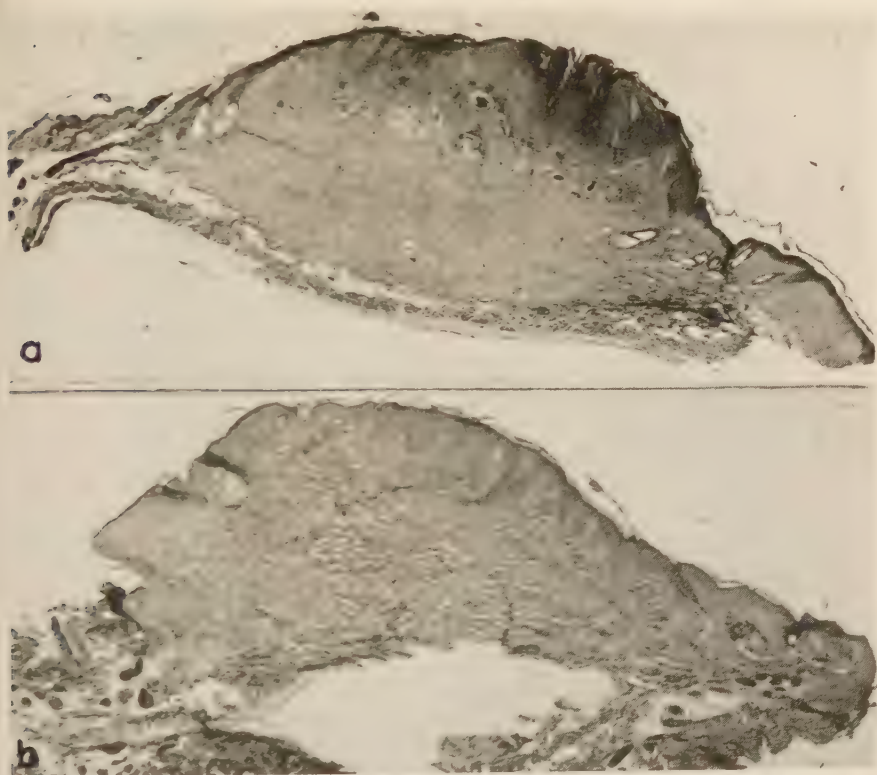


FIGURE 4. Benign neoplastic lesions in the site injected with vaccine virus in mice prepared with methylcholanthrene and cortisone. The lesion in FIGURE 4a ($\times 19$) is 52 days old from the day of the virus injection; that of FIGURE 4b ($\times 80$) is 94 days old.

tive than those in any other circumstances, but in their evolution and in their gross and microscopic features they seem to answer the essential requirements demanded by vaccinia infection; extracts of these mouse lesions injected in the skin of rabbits reproduce an infection seemingly identical to that currently induced by the rabbit-passage virus. The virus can be maintained also, but apparently less successfully, in passages through mice treated with MC alone or with cortisone alone. On the other hand, the virus cannot be maintained (or can be maintained only for a short number of passages) through untreated mice.

No further conclusions can be drawn from this work until more experiments—now under way—provide an answer to questions regarding such points as the nature of the agent maintained in the passages; the changes in infectivity of the agent for rabbits and the changes in the inflammatory and cell-stimulating power for mice with advancing mouse passages; and the dependence of the agent on treatment of the mice with both MC and cortisone. These points may be of great importance in trying to understand the phenomena I shall now describe.



FIGURE 5. Two stages—35 (5a) and 63 (5b) days—in the evolution of an epidermoid carcinoma in a mouse prepared with methylcholanthrene and cortisone and injected with vaccine virus. The derivation of the neoplasm from the scar is clearly manifest in FIGURE 5a.

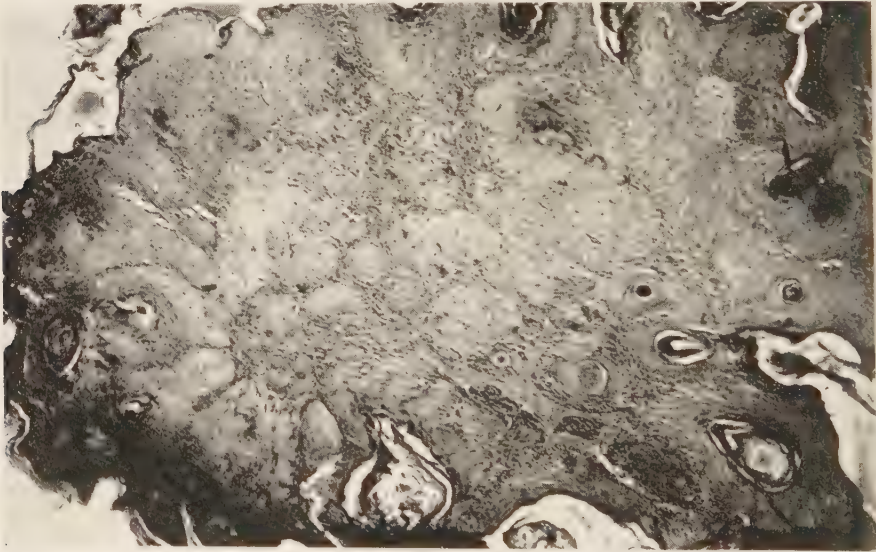


FIGURE 6. Epidermoid carcinoma in the mouse of FIGURE 5. $\times 60$.

As stated before, after the healing of the lesions induced by the rabbit virus, a large scar remained in those mice that had been prepared with MC and cortisone (FIGURE 3) — and also with cortisone alone — while in the other groups the scars were either much smaller or unnoticeable. It was in the mice treated with MC and cortisone that the central observation of this study was made, for it was exclusively in these mice, anywhere from three

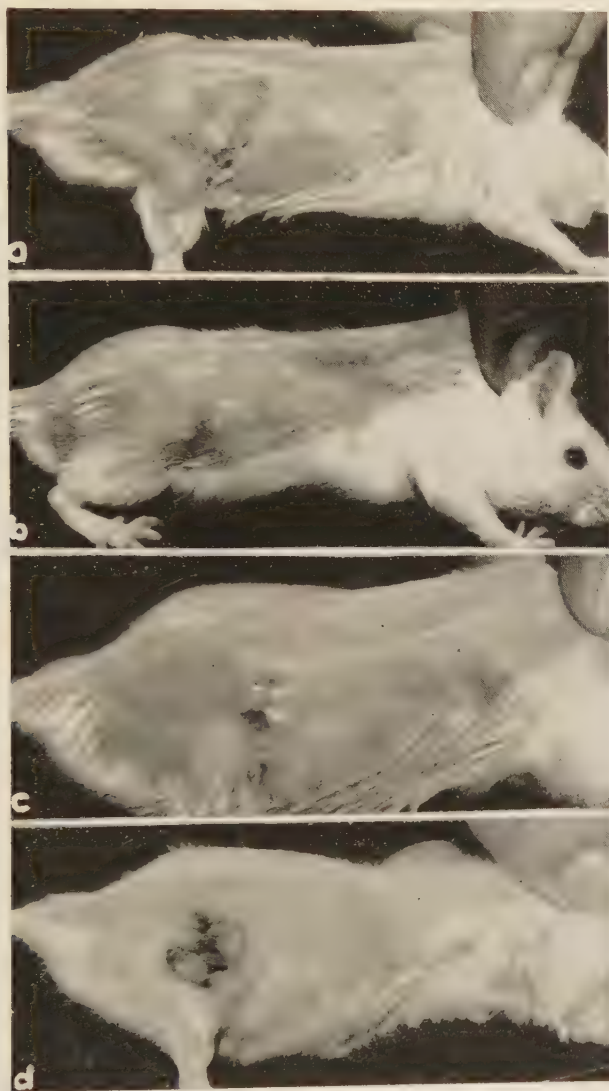


FIGURE 7. Evolution of a lesion—7 days (7a), 27 days (7b), 5 months (7c), and 6 months (7d)—from acute vaccinia infection to a sarcoma in a mouse treated with methylcholanthrene and cortisone.

weeks to a few months after the virus infection and strictly limited to the virus-infected site clearly revealed by the scar, that signs of growth became manifest.

In some cases these growths were benign fibrous or papillomatous structures that could regress after a few weeks of growth (FIGURE 4). In other cases, an initially mild lesion grew steadily and became, in a matter of weeks, an epidermoid carcinoma (FIGURES 5 and 6). In still other cases the lesion apparently remained benign for several months; in such cases the lesion

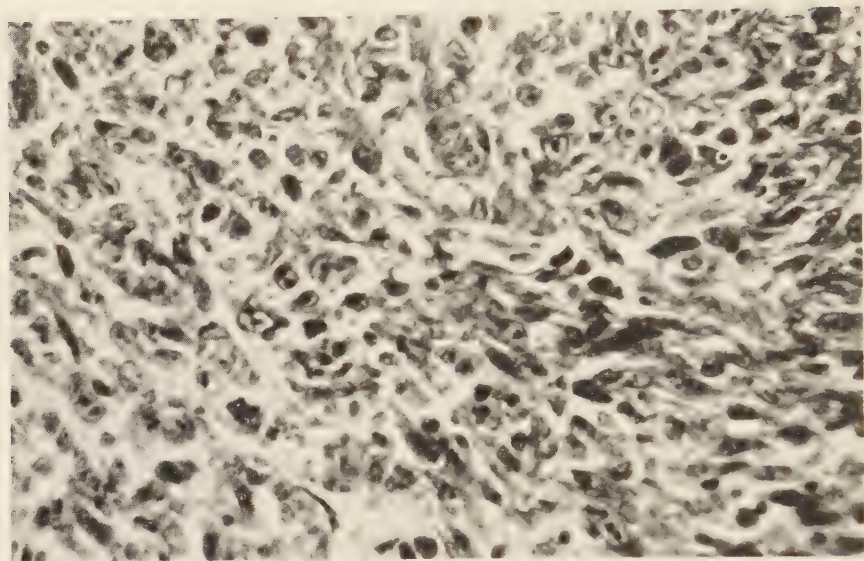


FIGURE 8. Sarcoma in the mouse of FIGURE 7. $\times 450$.

became a sarcoma (FIGURES 7 and 8). Two of these sarcomas became readily transplantable.

I shall now summarize the results obtained in a few representative experiments. To simplify, I shall call the growths that develop at the site injected with vaccine virus "site tumors," and those that develop at random away from the site of the virus injection in the dermal areas painted with MC I shall call "random tumors."

In one experiment, of 18 mice, 13 (72 per cent) developed site tumors, benign in 7 cases and malignant in 6. Regressions have occurred in 5 of the benign tumors, with possible regrowth in 2 of them. Random malignant tumors have occurred in 3 cases (17 per cent), coinciding in 1 mouse with a benign site tumor. Several mice of this experiment are still alive, and the above data may be altered.

In a second experiment only benign tumors have developed thus far in 10 mice; 8 are site tumors and 2 are random tumors, also benign and in both cases coinciding with a site tumor. Most of the mice of this experiment are still alive.

In a third experiment 1 benign and 5 malignant site tumors developed in 8 mice without any random tumors. This experiment was terminated 52 days after virus injection, so that random tumors may have developed at a later date.

The over-all results thus far available from these and from still another experiment are as follows: of a total of 42 mice, 28 (66 per cent) developed site tumors, 16 benign and 12 malignant; 8 of these mice (19 per cent), have developed random tumors, 4 benign, and 4 malignant.

These results are being fully confirmed in experiments under way with

neurovirus in Swiss mice and probably in mice from some inbred strains. Also—and this may be more significant—infection with dermovirus, a variant far less inflammatory and virulent than the neuro strain, seems to result in the development of site tumors more frequently and rapidly than with the latter strain.

On the other hand, tumors at the site of the vaccinia lesion have not yet developed in a total of 295 mice variously treated with the following: MC alone; MC plus EB; EB alone; EB plus benzol paintings; cortisone alone; cortisone plus benzol paintings; benzol paintings alone; nor, of course, have they developed in normal mice. Nor did site tumors develop in groups of mice treated as above, but injected with heat-killed virus and with benzol, which induces ulcers grossly similar to those induced by the virus in mice treated with MC plus cortisone.

A number of random tumors have appeared in all of the mice treated with MC either alone or in any combination, with or without virus. At present, however, we cannot give an estimate of the incidence of these tumors because the number varies from group to group, suggesting that each of the hormones and also the virus, or both in combination, may influence the number and type of these tumors; such an estimate requires careful analysis. This may likewise be true of a number of internal tumors, for example, in the lungs and lymph nodes, that have developed in mice painted with MC. No tumors of any kind have developed in mice treated with either of the hormones alone or with benzol, or with benzol alone.

It appears, then, that, subsequent to an acute virus infection, tumors arise in the precise site of the virus lesion, although in our experiments this has happened only in mice prepared with MC and cortisone.

Now the following questions should be asked: is the virus *itself* instrumental in the development of these tumors? If so, in what capacity? As a specific tumor agent undergoing (on a very specially prepared soil) a change comparable, as some but not all workers believe, to the change that takes place in the Shope virus in the papilloma-cancer sequence in the rabbit? Or as a nonspecific agent that, through the injury followed by repair, *promotes* the development of potential malignancies already *initiated* by MC? Is there present in the rabbit testes another agent instrumental in the effects described?

What, then, is the part played by cortisone? Is it purely to induce larger lesions, thus magnifying the injury-repair effect? To complement in other ways the effect of MC? To interfere with the full development of immune reactions of the host against the virus?

Obviously, no answers to these questions can be given at the present time. In the search for such answers we must (1) complete and extend our initial observations with other variants of vaccinia and other viruses (for example, herpes) infecting the skin and other tissues (especially lung) of different strains of mice and other animal species, and also investigate the possible effect of agents, viral or otherwise, that may accompany vaccinia in the rabbit testes or mouse skin; (2) ascertain whether a vaccinia antigen is present in transplants of the tumors that develop at the site injected with

vaccine virus, and investigate the effects of previous immunization of mice against vaccinia on the development of these tumors; and (3) attempt, in the course of the mouse passages or by other means, to isolate from vaccine virus variants that, in normal mice and or other species, induce lesions progressively more proliferative than inflammatory that may culminate in lasting neoplasia.

Summary

The intradermal injection of a suspension of vaccine virus from infected rabbit testes into Swiss mice prepared by skin paintings with MC combined with injections of cortisone, results in the development of a much enhanced dermal infection. Frequently this is followed by the development of a variety of both benign and malignant neoplastic lesions arising strictly from the precise site (still evident from the scar) injected with the virus material.

These neoplastic lesions have never developed following injections of the virus material into mice prepared (1) only with MC; (2) only with cortisone—in which hosts the infection is also enhanced; and (3) with EB with and without MC—in which hosts the infection is suppressed; not to mention normal mice.

The significance of these findings is briefly discussed.

Acknowledgment

This study has been carried out with the able assistance of Phyllis Johnson and Barbara Stanley.

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Part IV. Fowl Tumors

ROUS SARCOMA VIRUS: THE PURIFICATION PROBLEM

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Definition of the Problem

As may be surmised from the title of this presentation, the Rous sarcoma virus has not yet been isolated in sufficient amount, or in a state of sufficient purity to permit definitive studies to be made of its physical and chemical properties. The subject chosen for this discussion is, therefore, the nature of the purification problem itself; that is, the reasons for the failure of efforts made to purify this particular virus, especially since many other animal viruses, including two that cause neoplasia,¹ have been purified in appreciable quantities and to a fairly high degree.

This disease-producing agent was among the first "filterable viruses" to be discovered, having been reported by Rous² in 1911. Also, it was early recognized that this virus, which was capable of inducing cancer in chickens, was a potentially valuable laboratory tool for investigating both the nature of cancer and the mechanisms of neoplastic transformation. Many attempts have been made, therefore, not only by the earlier investigators who worked with this virus, but also by a succession of subsequent workers, to isolate and concentrate it in pure form. The various principles and methods employed in attempts to purify the Rous sarcoma virus were reviewed by Harris³ in 1953 and by Oberling and Guérin⁴ in 1954. The methods include most of the principles that have been applied successfully to the purification of other active biological substances, including enzymes, nucleoproteins, and numerous viruses of infectious diseases. A summary of the various principles and of the authors who have reported upon them is given in TABLE 1. Used alone, none of these principles has served to accomplish more than a partial separation of the virus from other nonviral cellular material. The most efficient methods, that is, in terms of total yield of virus as well as in progress toward purification, have involved combinations of two or more of the different separative procedures. Some examples are: (1) the combined use of filtration and differential centrifugation, by Claude;⁵ (2) the use of these same procedures together with enzymatic degradation of protein impurities with trypsin, by Amies and Carr;⁶ (3) the improved method of Carr and Harris⁷ involving the degradation of polysaccharides with hyaluronidase and of proteins with trypsin, followed by differential centrifugation in an ultracentrifuge; and (4) the method of Moloney⁸ which, in addition to the foregoing procedures, included precipitation of the biologically active fraction with protamine sulfate prior to tryptic digestion.

Although such methods have resulted in considerable increases in the relative purity of the final fractions as compared with the starting extracts

TABLE 1
SUMMARY OF PRINCIPLES OF PURIFICATION
APPLIED TO THE ROUS SARCOMA VIRUS

Purification principle	Materials and procedures
Adsorption	<i>of agent (and elution)</i> Kaolin ³⁷⁻⁴² Alumina gel ⁴⁰⁻⁴⁹ Diatomaceous earth ⁵⁰⁻⁵³ <i>of impurities</i> Charcoal ^{37, 39} Kaolin ³⁷⁻³⁹ Alumina gel ^{43, 47, 49, 54-57} Diatomaceous earth ^{37, 39} (also see Filtration)
Filtration	Sand and paper pulp ⁵⁷⁻⁵⁹ Diatomaceous earth ⁶⁰ Earthen candles (Berkefeld type) ^{2, 58, 59} Collodian membranes ^{40, 61-64}
Precipitation	<i>of agent</i> pH adjustment ^{6, 7, 25, 48, 64-66} Ammonium sulfate ⁶⁷⁻⁷⁰ Papain ^{3, 7, 71, 72} Histone ⁷¹ Protamine ^{8, 73} Methanol ^{3, 7} Metal buffers ^{74, 75} <i>of impurities (carbohydrate)</i> Gelatin ⁵⁵
Dialysis	Collodion bag ^{49, 56, 57, 67}
Extraction	Serial ^{54, 76} Differential (buffer-fluorocarbon) ⁷⁷
Degradation of impurities	Mucolytic enzymes ^{3, 7, 8, 34} Proteolytic enzymes ^{3, 6-8, 25, 47, 78, 79}
Centrifugation	Differential ^{5-8, 24, 73, 80-90} In density gradient ¹¹ At isodensity (clarification) ⁹¹

of tumor tissue, there has been no basis, until recently, for estimating absolute amounts of virus, or the actual "degree of purity" of the final fractions in terms of the ratio of viral to nonviral material. Even now, it is possible to make only rough "order of magnitude" estimates of this ratio. Using the electron micrographic technique for counting the number of physical particles of virus in a suspension, Sharp and Beard⁹ estimated the particle content of one of our standard virus preparations, CT 632,⁸ to be "of the order of" 10¹⁰ per ml. (see appendix to this report, by Sharp and Beard, p. 454). This

TABLE 2
ESTIMATION OF QUANTITY AND RELATIVE PURITY OF ROUS SARCOMA VIRUS
IN A "PARTIALLY PURIFIED" PREPARATION

(A) Individual particle of Rous sarcoma virus

Measurement or estimate	Source or derivation	Result
(1) Diameter.....	Kahler <i>et al.</i> ¹⁰	89 m μ
(2) Density.....	Kahler <i>et al.</i> ¹¹	1.15
(3) Volume.....	Computed from 1, assuming a sphere	3.7×10^{-16} ml.
(4) Mass.....	Computed from 2 and 3	4.24×10^{-16} gm.
(5) Dry weight.....	Computed from 4, assuming 50 per cent hydration	2.12×10^{-16} gm.
(6) Nitrogen content.....	Computed from 5, assuming 8 per cent of dry weight	1.70×10^{-17} gm.

(B) Partially purified microsome fraction (CT 632)

Measurement or estimate	Source or derivation	Result (quantity contained in 1.0 ml.)
(7) Number of virus particles..	Sharp and Beard*	"order of" 10^{10}
(8) Total virus mass.....	Computed from 4 and 7	4.24×10^{-6} gm. (= 4.24 μ g.)
(9) Virus nitrogen (N _v).....	Computed from 6 and 7	1.70×10^{-7} gm.
(10) Total nitrogen (N _T).....	Moloney ⁸	2.1×10^{-5} gm.
(11) Ratio of virus nitrogen to total nitrogen.....	N _v :N _T	1:124 (= 0.8 per cent)

* See APPENDIX by D. G. Sharp and J. W. Beard, p. 454.

value represents the total count of all of the particles that could be considered to be virus, that is, an estimate of the *upper limit* of possible virus content rather than of the actual number of known virus particles. With the aid of this upper limiting value for the absolute quantity of virus, it was possible to derive a rough estimate of the greatest possible degree of purity of the virus in this particular preparation, as illustrated in TABLE 2. The estimates of unit particle diameter and of density shown in section A of this table are those of Kahler *et al.*^{10,11} based upon quantitative measurements of tumor-producing activity. The assumed values for hydration (50 per cent) and nitrogen content (8 per cent) of individual Rous sarcoma virus particles are representative of the respective results that have been published for other viruses having similar size and density and that have been purified and characterized in detail.¹² Any errors introduced by inaccuracies of these assumed values are surely less than a factor of 2 and are probably negligible in comparison with the error of estimate of the particle count.

As is shown in the last row of TABLE 2 (section B), the ratio of virus nitrogen in this particular virus preparation was estimated to be, at best, 1:124, or only 0.8 per cent. If this ratio is taken as an approximate representation of the ratio of virus to total nitrogenous material, it is apparent that, in comparison with an overwhelming amount of "impurities," the total mass of virus is insignificant. Since the virus preparation represented (CT 632) was among those having the highest activity per unit of nitrogen of the materials thus far investigated in this laboratory, it is unlikely that the ratio of virus to "impurities" of approximately 1 to 100 has ever been appreciably exceeded.

This finding, although discouraging, was not at all unexpected in view of corroborating evidence of other types, and also in view of the failures of many investigators to isolate this agent by methods applicable to other viruses. Chief among the corroborating observations are the low frequencies of Rous sarcoma cells that can be shown to contain virus or viruslike particles when viewed under the electron microscope.^{3, 4, 13-16} Thus, Bernhard, Dontcheff, Oberling, and Vigier¹⁴ observed particles in only 5, or 0.13 per cent, of the 3,800 cells they examined. A similar average result was obtained by Epstein¹⁶ who, however, demonstrated a highly significant correlation between extractable virus activity and the frequency of particle-containing cells. In studies on 5 different lines of Rous sarcoma growing in ascites form and involving an examination of more than 15,000 cells, Epstein reported frequencies ranging from only 1 particle-containing cell per 3000 for the line having the weakest activity to 1 per 57 cells in one of the lines showing the highest amount of extractable virus. A slope of, essentially, unity for the curve relating the log TD_{50} infectious units and the log number of particle-containing cells per unit volume of cell suspension strongly indicates that the observed particles may indeed represent unit particles of the virus.

Similar conclusions with respect to a low virus content of Rous sarcoma cells have also been reached by other methods of approach. Thus, Carr,¹⁷ assuming that an infectious dose of the virus represented one physical particle, calculated the yield by the best methods of extraction as only 5 particles per 100 tumor cells. Commenting further upon Carr's calculation, Oberling and Guérin⁴ point out that "even if the minimum infectious dose is taken as 1000 particles, the number of virus particles in one cell will not exceed 50, which leaves little hope of demonstrating their presence by use of the electron microscope." In this connection it is of interest to point out that the particle count of 10^{10} per ml. found for standard preparation CT 632 (TABLE 2) and the average inoculum yielding a 50 per cent response in New Hampshire Red chickens (that is, 0.2 ml. of the $10^{-6.5}$ dilution) lead to an estimate on an average of 630 virus particles per 50 per cent infectious unit of this material in the particular strain of chicken employed. This, however, represents an estimate of the maximal number that could be present, rather than the number of known virus particles (see above).

Finally, in kinetic studies on Rous sarcoma cells and causative virus in a tissue-culture system, Rubin,¹⁸ estimated that only 1 cell out of 35 to 60 produced virus within a 6-hr. period and that the most probable number of particles released per virus-producing cell during this time was 5. This led

him to conclude that "all the cells release virus in a slow trickle," and that "the association between cells and virus is stable, since neither component outgrows the other to any measurable extent." Like the calculations of Carr,¹⁷ those of Rubin,¹⁸ with respect to absolute numbers of virus particles, were based upon the assumption that one physical particle constitutes a biological infectious unit; as his criterion of viral activity, however, Rubin used the count of local ectodermal lesions on chick membranes instead of the 50 per cent incidence response. Even on allowing a factor of as much as 1000 for a possible error of the assumed plating efficiency, however, not more than about 30 particles per cell would be expected at any given time, according to the results published by Rubin.

It is apparent that the evidence obtained with all quantitative approaches available at the present time leads to the same general conclusion; namely, that the amount of Rous sarcoma virus in tumor cells, or in extracts of them, is extremely small in relation to the total quantities of other nonviral cellular constituents.

Methods of Approach

The findings described thus far, together with repeated failure to isolate the Rous sarcoma virus in practical working quantities by methods applicable to other viruses, led to a reorientation of the efforts directed toward this end. Three lines of approach are now being followed. The first two are directed toward increasing the amount of virus recoverable from extracts of source tumor tissues. The third represents a continuation of efforts to facilitate separation of the virus by various purification methods through enzymatic degradation of complex, nonviral biological substances, or "impurities." The respective lines of approach and some recent results obtained with them are summarized in the following sections.

Attempts to increase virus concentration in tumor tissue by biological methods. Previous studies had shown that the amount of virus extractable from Rous sarcomas was related to the dose of virus used to initiate the tumors.¹⁹ When the strongest initiating doses were used the amount of virus in the extracts was slightly higher, on an average, than in the standard virus inocula. Also, the individual tumors varied considerably in their yields of virus, and extracts obtained from some of them were up to 10 or 20 times more potent than the strongest initiating doses. These findings suggested that the virus content of starting extracts might be regularly increased by a factor of about 10, provided a method could be found for selecting those tumors that, according to chance, contained the higher concentrations of virus.

Also, the possibility exists that the greatest concentrations of virus in tumor tissue now observed may not represent ultimate biological limits. Still higher concentrations might be achieved through selective serial passage of the virus in chickens; for example, by using the most potent available virus suspension for each successive passage.

The results of experiments carried out for the purpose of testing the foregoing possibilities are summarized in FIGURE 1. To start the passage series, 10 chickens were inoculated in the wing web with the 10^{-2} dilution of a frozen

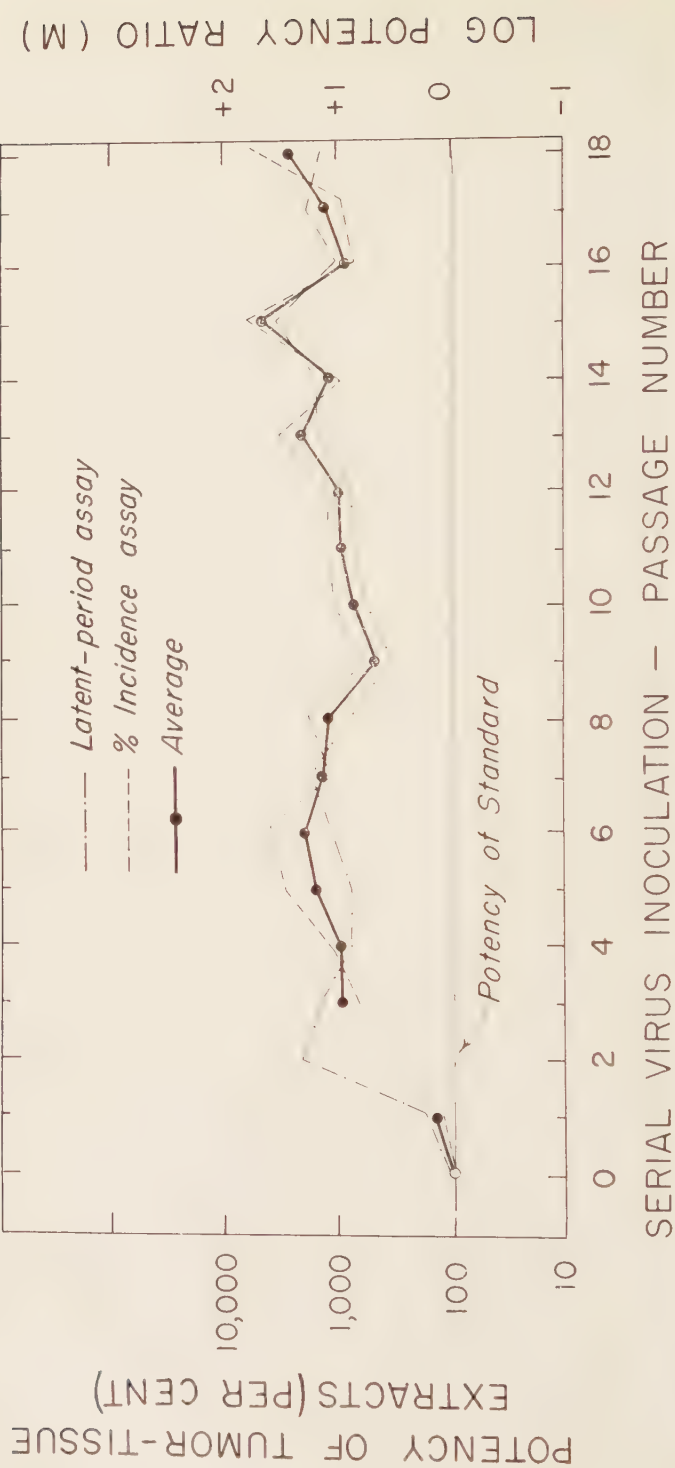


FIGURE 1. Relative potencies of extracts of the most rapidly growing tumors at successive serial passages of the Rous sarcoma virus in chickens; referred to frozen standard-virus preparation CT 629 (= 100 per cent).

standard preparation of Rous sarcoma virus (CT 629).⁸ The chicken having the largest tumor 8 days later was sacrificed, and its tumor was taken for further passage of the virus. The tumor was extracted by procedures previously described,¹⁹ and serial tenfold dilutions of the extract (10^{-2} through 10^{-9}) were prepared and inoculated into groups of 10 chickens (cf. Bryan²⁰). For further passages of the virus the most rapidly growing tumor of the group induced with the strongest dose of extract (10^{-2}) was selected in each successive assay series.* Bioassay results were interpreted from both the latent period and percentage-incidence data,¹⁹⁻²¹ except in one experiment (the second passage) in which dilutions of the "unknown" (extract) higher than 10^{-6} were not tested.

As is shown in FIGURE 1, extracts of tumors selected after the first virus passage were, on an average, more potent by a factor of about 10 than the standard virus preparation. The standard (CT 629) used in this study was practically identical in potency (cf. Moloney²²) with that (CT 559) employed in earlier investigations,¹⁹ in which extract potencies as high as those shown here were obtained only infrequently as a result of chance variation. It is apparent, therefore, that selection of the most rapidly growing tumor (or tumors) as a source of virus resulted in yields of extractable virus about 10 times greater, on an average, than those obtained without such selection.

The findings were less encouraging with respect to an over-all increase in virus content. The very slight upward trend of the potency values (after the second passage shown in FIGURE 1) is not statistically significant. There is accordingly no evidence as yet to show that this method makes it possible to increase the virus content of tumor tissue beyond the presently observed limits. Nevertheless, through further serial passage of the virus, efforts in this direction are being continued. Similar studies involving serial passage of the virus in the brains of young chicks²² and on the membranes of developing chick embryos are in progress in the laboratory of Vincent Groupé, Rutgers University, New Brunswick, N. J. (personal communication). Other systems, using tissue culture, that might favor the accomplishment of this important objective, should also be tried.

Elucidation of factors associated with the lability of the virus. It is well known that with respect to its lability under some laboratory conditions,²¹ the Rous sarcoma virus is highly variable, and that many preparations lose potency rapidly after "partial purification" by any method.³⁻⁵ One of the factors that might be associated with this variable stability became evident in studies²³ made in collaboration with M. E. Maver on the comparative biological activity of the virus at different levels of pH in citrate and in phosphate buffers. Whereas the results obtained with the former buffer were uniform and entirely reproducible, those obtained with the latter were highly variable and unpredictable.^{24, 23} Further consideration of the phenomenon of greater stability in citrate buffers led to investigations on the oxidation of lipid components of the tumor and its microsome fraction. It

* In many experiments several of the tumors were equally large on the sixth to eighth day and no particular one could be singled out as being the largest. In such cases one was taken at random.

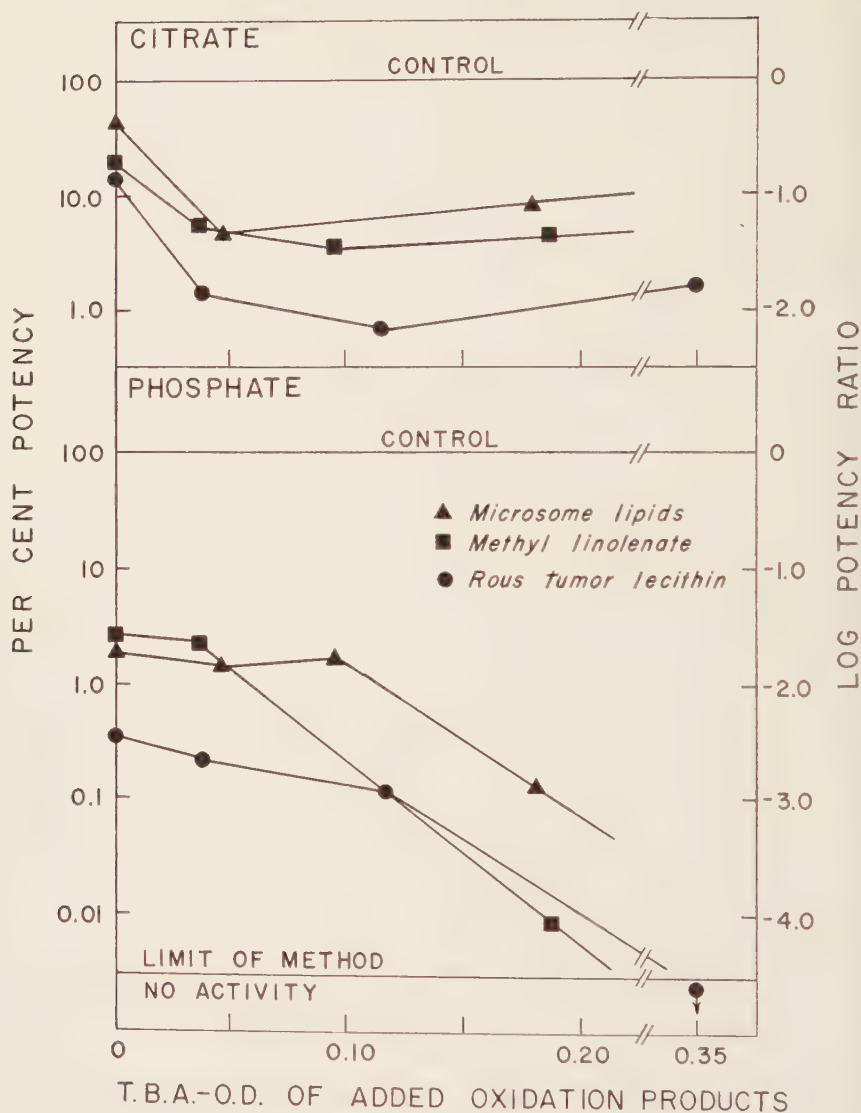


FIGURE 2. Comparison of residual potencies of constant amounts of standard Rous sarcoma virus in citrate and in phosphate buffers, after treatment with water-soluble oxidation products derived from various sources. The concentrations of added oxidation products are expressed as thiobarbituric acid optical-density values (TBA-O.D.).

was known, for example, that active fractions of Rous sarcoma are very high in lipid content (35 to 53 per cent),^{3, 24, 25} and that the oxidation of lipids may be inhibited to some extent with citrate reagents (for example, see Kuck²⁶). Also, other investigators had shown that oxidized unsaturated fatty acids were capable of inhibiting cell division,²⁷ the enzyme activity of mitochondria,²⁸ and respiration and glycolysis in Ehrlich ascites cells.²⁹

FIGURE 2 shows some results of recent experiments in which aqueous extracts of the oxidation products of Rous tumor lecithin and of the microsome lipids (phosphatides with the properties of a lecithin) were tested for their ability to inactivate standard preparations of the Rous sarcoma virus. For comparison, results are also shown for a similar extract of oxidized methyl linolenate, an ester of an unsaturated fatty acid that may be considered as representative of the component of lecithin that gives rise to the reactive oxidation products. Oxidation of the lipid fractions and of methyl linolenate was carried out under controlled conditions with ultraviolet light. Concentrations of the products of fatty-acid oxidation in the aqueous extracts were measured by the thiobarbituric acid (TBA) reaction of Kohn and Liversedge.³⁰ Aliquots containing the desired amounts of the products for use in the experiments were prepared by appropriate dilution with triple distilled water. The adjusted aliquots were mixed with constant amounts of a 10^{-2} dilution of a standard virus suspension (CT 632⁹) in either citrate or phosphate buffer (pH 6.0; 0.05 M) and were incubated for a period of 75 min. at 37° C. after which they were tested for residual biological activity by methods previously described.¹⁴ Incubated controls in which only water was added to the buffer suspensions of virus are represented by the points plotted at zero concentration of the added product (in terms of TBA absorption values at 530 m μ) in FIGURE 2. The horizontal line at 100 per cent in each chart represents the uninoculated control, which was a freshly thawed and diluted sample of the same frozen standard virus as that used in the experiment*.

As may be seen in FIGURE 2, the water-soluble products of fatty-acid oxidation (as measured by the TBA reaction) were capable of inactivating Rous sarcoma virus in phosphate buffer in relation to their concentration. On the other hand, citrate salts protected the virus from the inactivating effect of similar concentrations of the added oxidation products. However, the protective action of citrate in the amounts used here can be overcome by still higher concentrations of the oxidation products.

It is hoped that this and additional information on factors that influence stability will lead to better methods of preventing oxidation and, thereby, to recoveries of larger amounts of the virus from presently available virus source materials.

Studies on Methods of Dissociation of the Virus from Preponderant Amounts of Nonviral Material

It is not without significance that the only two tumor viruses that have been purified to an appreciable extent were found to be present in extremely high concentrations in the infected tissues or fluids from which they had been separated. Thus, Beard and his associates^{1, 31, 32} found virus-particle concentrations as high as 2×10^{12} per ml. of plasma derived from chickens diseased with myeloblastosis; and Beard³³ estimated that the amount of virus recoverable from the most active papillomas of cottontail rabbits was about 18×10^{12} particles per gm. of wart tissue.

* Further details and additional data will be presented in a report to be published elsewhere.

However, the fact that an extremely high concentration of virus may not have been the only factor responsible for successful purification of the myeloblastic leukemia and papilloma viruses is suggested by another important way in which the source materials for these two agents differed from those of the Rous, as well as other tumor virus materials. In the former instance, the starting material subjected to further purification was a noncellular fluid (plasma) that normally contains relatively little particulate matter in the virus size range (that is, in relation to 10^{12} per ml. of viral particles). In the latter instance the clarified extracts of highly keratinized papillomatous tissue contained relatively little cellular particulate matter other than colloidal bits of insoluble keratinous material, which could be separated readily during several cycles of alternate high- and low-speed centrifugation.

It is possible that, if Rous sarcoma virus can be sufficiently increased in the source tissues or extracts, as suggested above, purification of this agent might be accomplished by the same methods successfully applied to the other tumor viruses (filtration and differential centrifugation). On the other hand, there is no definite indication at the present time that it can be so increased, and the strongest concentrations now observed therefore may represent the maximum biological limits for this agent. For this reason, systematic investigations on the degradation of nonviral biological substances, or other methods of facilitating the dissociation of virus particles from preponderant amounts of "impurities," seem to be strongly indicated as aids to further purification.

The use of mucolytic enzymes for reducing viscosity and facilitating the separation of virus from extracts of the highly mucinous Rous sarcoma was originally introduced by Claude.⁴¹ With the advent of commercial sources of hyaluronidase, the use of this mucolytic enzyme has become an important preliminary step in recent purification procedures.^{7, 8}

Trypsin has also been used with some success, at room temperature, for the degradation of nonviral protein impurities^{6, 7} or for the dissociation of the complex formed by precipitation of the active portion of microsome fractions with protamine sulfate.⁹ At the higher incubation temperature of $37^{\circ}\text{C}.$, however, the biological activity of the Rous sarcoma virus may be entirely destroyed by this proteolytic enzyme.³⁵

Microsome fractions prepared from Rous sarcoma tissues by the method of differential centrifugation have a total pentosenucleic acid (PNA) content of about 5 to 10 per cent, as estimated from published nitrogen and phosphorus values.^{8, 21} This represents a considerably larger quantity (perhaps 100 times more) than might be considered a content of the virus particles themselves; that is, if the calculations of TABLE 2 are to be accepted. Ribonuclease is therefore another enzyme that may be useful as an aid to purification. Preliminary studies in this laboratory indicate that there is no loss in biological activity of microsome fractions ("T-2" preparations of reference⁸) treated with this enzyme under conditions that cause it to hydrolyze about 75 per cent of the total PNA as measured by the release of phosphorus. The extent to which treatment with ribonuclease may aid further purification

has not yet been determined. The high phospholipid content (35 to 53 per cent) of the microsome fraction²⁴⁻²⁵ suggests that lecithinases or esterases may also be of potential value in isolating the Rous sarcoma virus.

The degradation of impurities by chemical substances other than enzymes is also to be considered. One such substance, sodium deoxycholate, which has been found to be of value in the separation of liver cytoplasmic ribonucleoprotein particles,²⁶ was tested in this respect. It was found, however, that this material completely inactivated the virus under the conditions employed.

Progress on the problem of dissociating the Rous sarcoma virus from excessive quantities of nonviral material is necessarily slow because of the extensive requirement in highly accurate bioassays (for example, for determining the conditions and concentrations of materials for optimal effects without decreasing the activity of the virus) and because of the complexity of available assay methods based upon chicken inoculations (see Bryan²¹ for review). It is possible, however, that future developments in bioassay methods may allow more rapid progress to be made in investigations of this general type.

Summary and Conclusions

In general, the relative ease or difficulty with which any virus can be separated from other components of source tissues derived from infected hosts is a function of: (1) the stability of the virus, or its ability to withstand various laboratory manipulations; (2) the nature and the degree of cellularity of the biological material employed as a source of the virus; and (3) the concentration of the virus in the source material, that is, the total mass of virus in relation to other extractable tissue components.

With presently available source materials (tumor tissue) the major problem in purification of the Rous sarcoma virus appears to be that of separating a relatively small amount of labile virus from overwhelming quantities of nonviral cellular constituents, or "impurities." The ratio of virus to impurities in the best "partially purified" preparations is estimated to be about 1:100.

Three lines of approach are being followed in efforts further to purify the virus under these conditions. They are: (1) attempts made by biological methods to increase the concentration of virus in the source tumor tissue; (2) elucidation of the factors concerned with the lability of the virus, with a view toward achieving its better preservation and a consequent increase in final yield; and (3) degradation of nonviral biological "impurities" by enzymatic action (or by other chemical or physical procedures) for the purpose of facilitating their separation through the use of established purification methods. Some results of recent investigations along these lines have been described.

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APPENDIX

ELECTRON MICROGRAPHY OF ROUS SARCOMA VIRUS PREPARATIONS*

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In the foregoing paper Bryan and Moloney have discussed the problems attending efforts to obtain the Rous sarcoma virus in purified preparations. These investigators also described biological attributes, particularly with respect to infectious activity, of preparations derived through studies over a period of years.^{1, 2} From the results of these investigations, and by means of a variety of assumptions, Bryan and Moloney have computed the approximate virus content of their final fraction of one specimen, CT 632,² which is, in effect, an estimate of the homogeneity of the preparation with respect to the agent itself. It is the purpose of this appendix to supplement the biological findings with the results of electron microscopic examination of the Rous sarcoma virus preparation.

Materials and Methods

The material employed for the study was the preparation CT 632, referred to in the preceding discussion and prepared as described in detail by Moloney.² It was received sealed in volumes of 0.15 ml. in glass ampoules and was kept frozen in crushed CO₂ ice. For an examination an ampoule was opened after rapid thawing of the contents, and the suspension was diluted 1:100 with a 0.14-M NaCl solution. The dilution was then spun in an ultracentrifuge cell designed³ for the sedimentation of virus particles onto an agar surface. After centrifugation at 16,300 g for 30 min., the agar block was removed from the cell in the usual way, the fluid was allowed to seep into the agar, and the material on the surface was fixed with osmic acid vapor. A pseudo-replica was prepared with collodion and was lightly shadowed with chromium.

Results

FIGURE 1, a picture obtained by this method, shows an array of particles of fairly uniform morphologic characters scattered, in relatively small physical amount, among masses of amorphous material. Some of the particles of interest are spheroidal in shape and entirely coated with chromium, and they show no evidence of intraparticulate structure. Others, however, are less well shadowed; these exhibit a differentiation in structure within the particle. There one may see evidence of a dense, elevated, centralized region bounded by clearer areas and a limiting membranelike structure. In addition, there are numerous images of the approximate size of the spheroids that have the appearance of empty membranes of the sort regarded as

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FIGURE 1. Preparation CT 632 of "purified" Rous sarcoma virus diluted 1:100 and sedimented onto an agar surface. $\times 16,900$.

"ghosts" in association with the particles of other viruses.^{4, 5} FIGURE 2a is a higher magnification of a small area of the micrograph of FIGURE 1.

That these particles represent the Rous sarcoma virus is scarcely to be doubted, although no quantitative studies have been made for the specific identification of the particles with the agent. Nevertheless, the high infectious activity of the preparation⁶ indicated a correspondingly high content of virus, and the fractionation sequence, as well as the conditions of sedi-

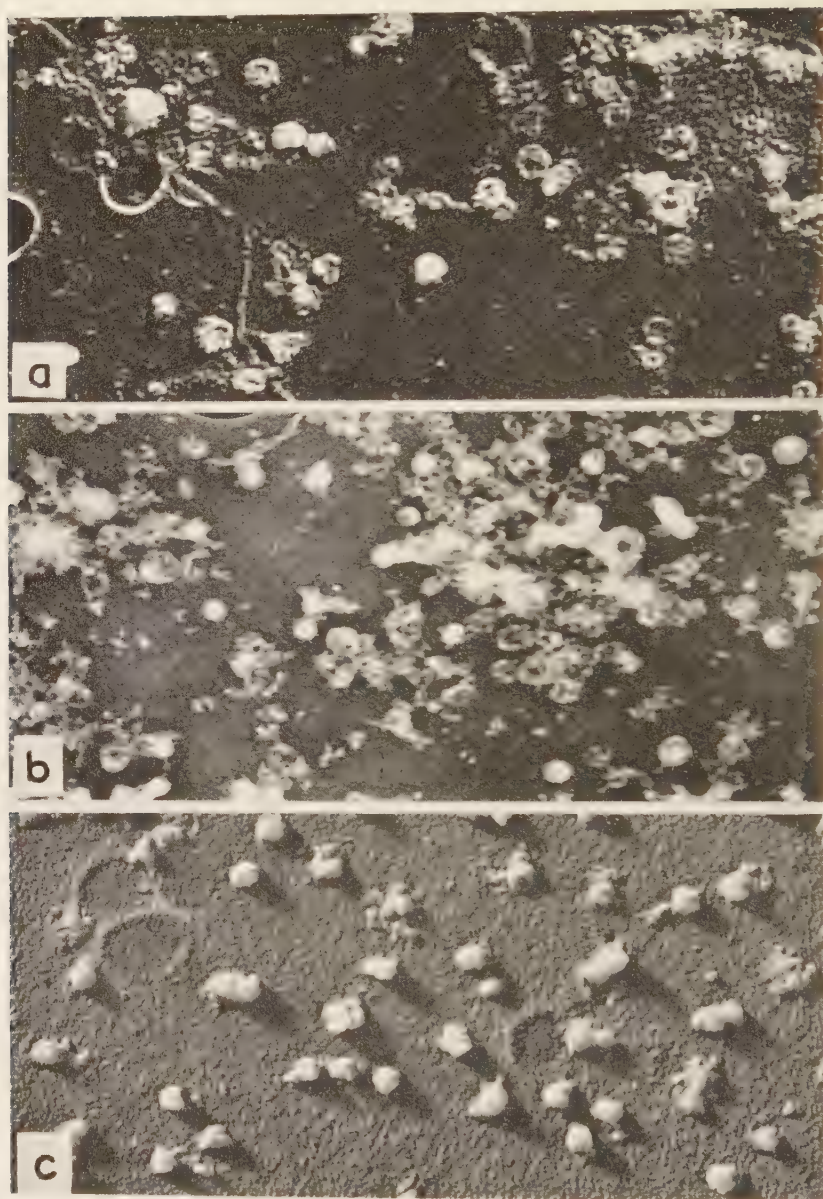


FIGURE 2. (a) A strip of the electron micrograph of FIGURE 1 at a higher magnification ($\times 34,000$); (b) virus of avian erythroblastosis in a concentrate obtained from chicken plasmas and *lightly* shadowed with chromium ($\times 34,000$); and (c) virus of avian erythroblastosis in a concentrate obtained from chicken plasma and shadowed with chromium in the usual way.

mentation for electron micrography, were compatible with recovery of the virus. Furthermore, the particles are of a size estimated⁶ for the Rous sarcoma virus and, finally, they are morphologically similar⁷ to the particles of another avian neoplastic disease, erythroblastic leukemia. This is obvious by comparison of FIGURES 1 and 2a with the micrograph of erythroblastosis virus shown in FIGURES 2b and 2c. The erythroblastosis virus of FIGURE 2b was very lightly shadowed, resulting in the display of internal structure and, as it happened, the presence of many ghosts. In FIGURE 2c the erythroblastosis virus in a concentrate obtained from chicken plasma was shadowed in the usual way. The identification of these particles with the virus has been established,⁸ and evidence of kinship of the agent with the Rous sarcoma virus has been described in Beard's article elsewhere in these pages.

It was evident from inspection of the electron micrograph of FIGURE 1 that the content of particles, presumably the virus, was very high. Counts of the particles yielded values indicating a level of the order of 10^9 particles per ml. The result suggests that the initial level in the source material must have been close to 10^{10} particles per gm. of tumor tissue since, on the basis of experience in the purification of other viruses, the loss must have been at least 90 per cent during the fractionation process.

Summary

The computations by Bryan and Moloney yielded a ratio of virus mass (nitrogen) to extraneous material (nitrogen) of about 1:100, indicating that the virus content of the preparation was of the order of approximately 1 per cent. The appearance of the electron micrographs is entirely compatible with this value. Despite the errors to be expected with such assumptions and the lack of accuracy of the particle counts, the value is one that may be judged to be fairly descriptive of the actual situation. Although it is clear that homogeneity with respect to virus has not yet been even remotely approached, nevertheless, the preparation gives indications of substantial progress in the physical isolation of the agent.

Broader implications of the findings with the electron microscope are those bearing on the results and interpretations of earlier studies on purified preparations of the Rous sarcoma virus. Attention has been directed before⁹ to the fallacies in the interpretations¹⁰ of the physical, chemical, and biological characteristics of the Rous sarcoma virus based on analyses of undefined tissue fractions, particularly such as those used by Claude.¹¹ A simple inspection of the electron micrograph of FIGURE 1, which was obtained with one of the best preparations, as judged by level of infectious activity, indicates clearly the complete lack of value of analyses on any material thus far described¹⁰ with respect to interpretations of the physical, chemical, and quantitative biological properties of the agent of the Rous sarcoma.

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THE PRODUCTION OF VIRUS BY ROUS SARCOMA CELLS

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Our understanding of the quantitative interrelationships between cells and certain viruses has increased spectacularly in the last fifteen years. This progress has been made possible through a combination of technical developments, such as precise assays *in vitro* and the one-step growth curve, which have permitted accurate measurement of the dynamic aspects of infection. Such precise information about the multiplication of tumor viruses has been markedly absent, however, a fact that can be attributed largely to the inadequacy of the virus-assay techniques. An assay technique meeting some of the basic requirements was developed for the Rous sarcoma by Keogh (1938). Virus concentration in a sample was estimated by the number of tumors produced by plating the sample on the chorioallantoic membrane (CAM) of the developing chick embryo. Although some workers have found this technique to be erratic with the strains of embryos and virus used in their laboratories, the virus strain that we used produced tumors on the CAM of our embryos with great efficiency. We have combined this technique with modern tissue-culture and cell-counting methods in an effort to define some of the parameters of cell infection induced by the Rous sarcoma virus. Particular stress has been placed on finding out the fraction of cells in a tumor that produces virus, and the amount of virus produced by the individual cells.

Much of this investigation has been fully reported elsewhere (Rubin, 1955, 1956), and these papers should be consulted for detailed information. I shall review briefly some of this published work, and also describe at greater length more recent unpublished work.

Methods

All experiments were carried out with Rous sarcoma cells *in vitro*. The cells were not permanently so maintained, however, but were passed serially every week in young chicks. The tumors so produced were removed and treated with hyaluronidase and trypsin prior to culture in monolayers on glass. Once the cells were established in culture, they could be easily suspended with trypsin alone (Dulbecco and Vogt, 1954).

The CAM assay was more sensitive than the endpoint titration techniques carried out in chickens in our laboratory. However, it agreed very closely with the average of several endpoint titrations carried out in more sensitive chickens by W. Ray Bryan. The CAM technique also proved the more accurate of the 2 assay methods, since the use of 5 embryos gave tumor counts with a standard error of only 30 to 50 per cent, when there was an average of between 100 to 300 tumors per CAM.

In our system the average number of tumors per CAM showed a linear

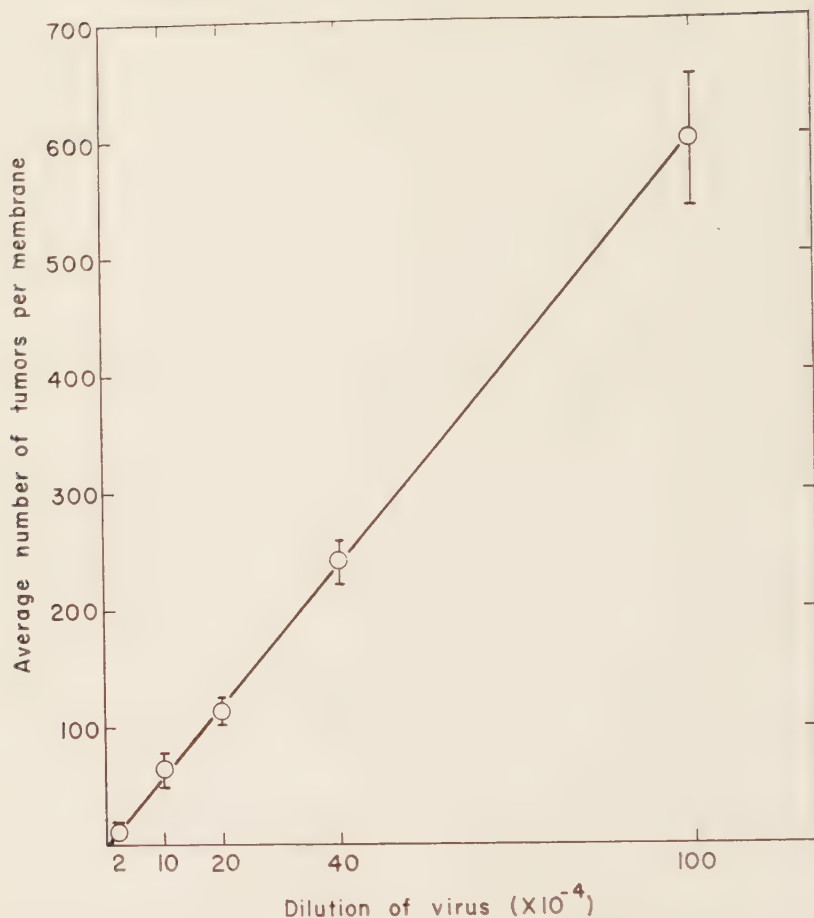


FIGURE 1. Relation between virus concentration and the number of tumors formed on the CAM.

dependence on the concentration of the inoculum, confirming Keogh's finding. This can be seen in FIGURE 1, where the concentration of inoculum is plotted on a linear scale against the number of ensuing tumors. Through analysis of the Poisson distribution, it can be proved that each CAM tumor is initiated by a single virus particle; thus, the virus found in each tumor represents a clone. It is likely that it represents a *pure* clone, since the number of infective particles that can be demonstrated in an ectodermal tumor is less than 1000, making it unlikely that a mutation should occur in every tumor.

In the case of the Rous sarcoma, this finding is of unique interest. At first, each CAM tumor is composed exclusively of ectodermal cells; yet, seven days after infection, malignant mesodermal proliferation can be seen at the base of each ectodermal tumor (FIGURES 2-5). These two distinct



FIGURE 2. Normal CAM.



FIGURE 3. Ectodermal tumor six days after infection of the CAM by the Rous sarcoma virus.

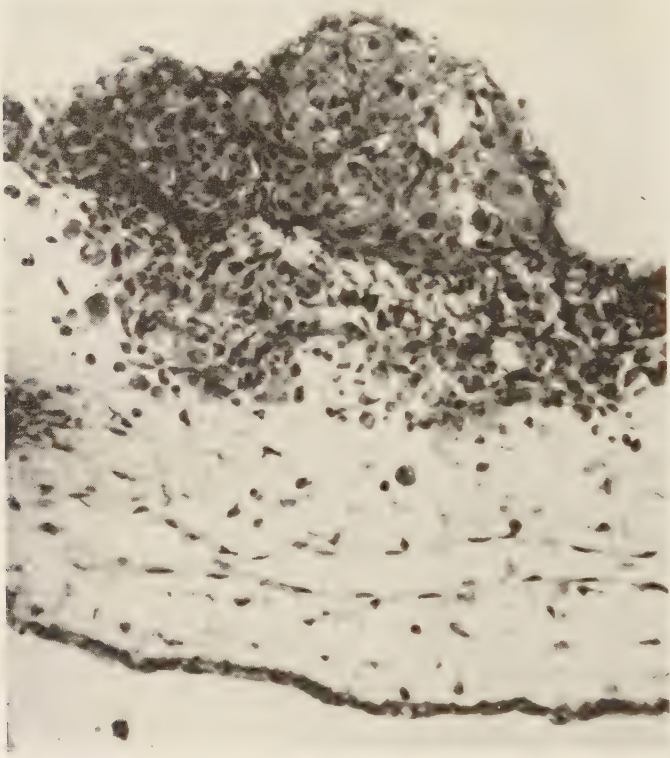


FIGURE 4. Early step in the invasion of the CAM mesoderm by infected ectodermal cells.

types of tumor cells are due to infection by the same virus. It has been shown that the virus that causes both tumors is the same, and has not undergone mutation (Rubin 1955). Thus, the type of tumor in the embryo depends only upon the type of cell infected. (The abbreviation t.f.u. will hereafter be used for tumor forming units and refers to the number of virus particles that can successfully initiate tumor growth on the CAM.

Rate of Virus Production in a Large Population of Sarcoma Cells

Having ascertained the various parameters of the assay system, we could study the rate of virus production by sarcoma cells in suspension. Cells were washed free of extracellular virus, placed in nutrient medium, and incubated at 37° C. Aliquots were taken at hourly intervals, the cells were spun down, and the supernate was assayed for virus. The results averaged from 7 experiments are presented in FIGURE 6. Extracellular virus increased at a linear rate up to 6 hours, when it reached a constant level. Since these cells were continually producing virus, the leveling off must have been due to a steady-state condition between virus production and thermal inactiva-

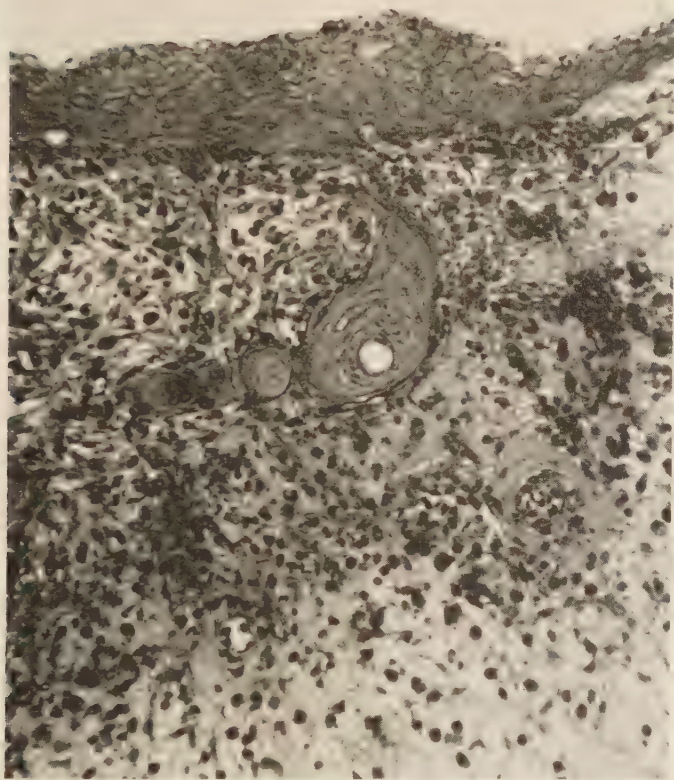


FIGURE 5. Tumor cells of ectodermal and mesodermal types multiplying side by side, both due to infection by the Rous sarcoma virus.

tion. Therefore, in order to find the true rate of virus multiplication, it was necessary to solve a differential equation that included terms for virus production and inactivation (Rubin, 1955). The solution showed that the rate of virus production could be readily calculated from the number of t.f.u. present in the medium after a constant level was reached (that is, after 6 hours), and the known rate of thermal inactivation (FIGURE 7) of the virus at 37°C . The calculated rate of virus production was 1 t.f.u. per 100 cells per hour (cells in cyanide did not produce virus).

During the course of 48 serial passages of the tumor in chickens, using tissue-culture cells derived from various passages, the rate constant for virus production was determined many times. Whenever tested under standard conditions in tissue culture, the cells from all passages produced virus at the same rate. This indicated that an intimate relationship existed between

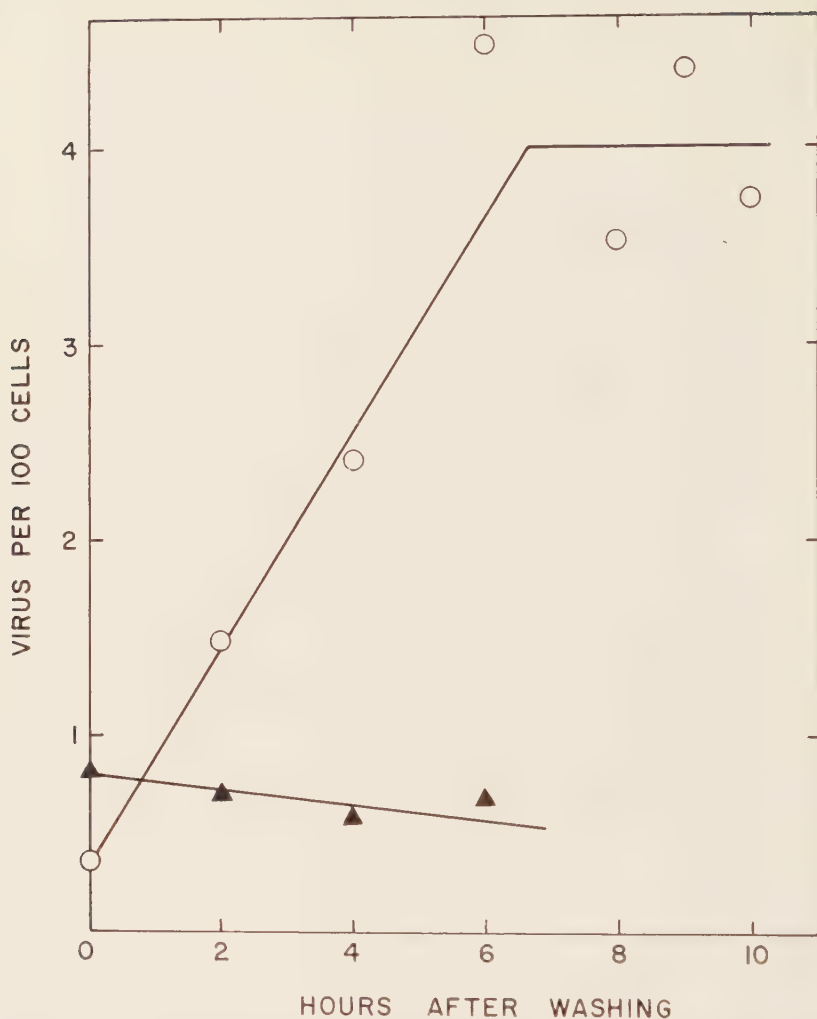


FIGURE 6. The symbols —○—○ indicate the virus produced into the medium by Rous sarcoma cells; —▲—▲, the virus produced by Rous sarcoma cells in the presence of KCN.

virus and cell, since neither component outgrew the other to a measurable extent.

Release Time of the Virus

In several of the growth experiments, intracellular as well as extracellular infectious virus was measured. Cells were fragmented by 3 cycles of rapid freezing and thawing, a procedure that did not inactivate virus when carried out in a medium that contained protein. The intracellular virus level remained constant throughout the growth experiment (about 1 t.f.u. for every 200 cells). If we assume that at any given time the increase in extracellular virus is proportional to the concentration of intracellular virus

present at that time, then the rate of increase can be represented by the differential equation

$$\frac{dV}{dt} = KIN$$

where V represents extracellular virus; t , time (minutes) after washing; I , intracellular virus per cell (constant in the Rous sarcoma); N , number of cells; and K , velocity constant for the transition $I \rightarrow V$ (min.^{-1}).

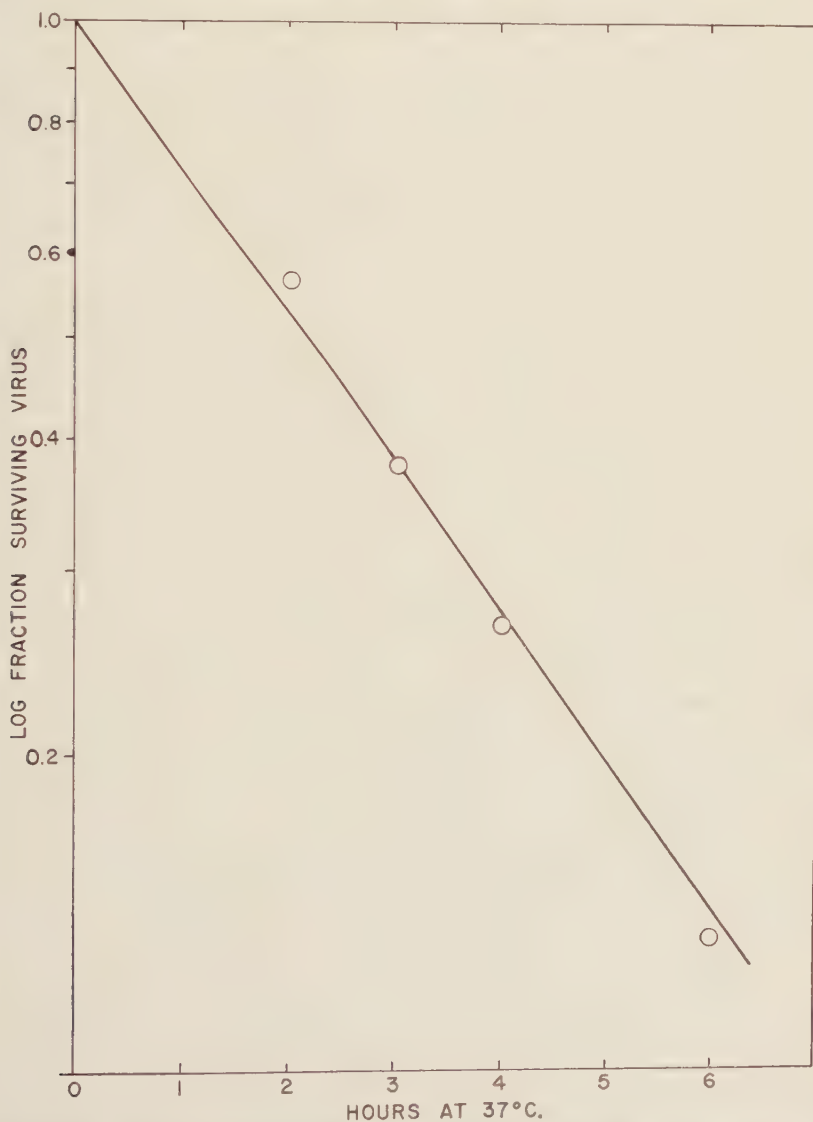


FIGURE 7. Kinetics of thermal inactivation of the Rous sarcoma virus in tissue culture medium at 37° C.

Upon integration, this equation can be solved for $\frac{1}{K}$, which is defined as the release time, or the time required for a virus, after it has become infective, to be released from the cell into the medium.

$$\frac{1}{K} = \frac{IN(t_2 - t_1)}{V_2 - V_1}$$

where V_1 and V_2 are the concentrations of extracellular virus at times t_1 and t_2 , respectively.

When the experimental values are substituted, the release time is found to have a value of about 30 minutes. In other words, the average Rous sarcoma virus particle stays within the cell for one half-hour after its completion. By contrast, Western equine encephalomyelitis and Newcastle disease viruses have release times of only a few minutes (Rubin *et al.*, 1955; Rubin and Franklin, unpublished data), while the time for poliomyelitis is several hours (Howes and Melnick, personal communication).

Virus Yield by Enlarged Cells

Until now we have considered virus production by cells under standard conditions. The cited cells were about 13μ in diameter and had never been under cultivation for more than a few days. Repeated attempts to obtain multiplication of these cells failed. The cells continued to grow in size, however, and it was noted that, as the cells enlarged, the amount of virus in the medium increased. The following experiments were carried out to study the relationship between cell size and virus production. Ten to 15 cultures were started, and every 2 to 3 days the medium was changed and assayed for virus. At each fluid change, 2 of the cultures were suspended and the cells counted. The average diameter of the collected cells was determined by measuring 40 to 60 cells with an ocular micrometer (the cell volume was calculated on the assumption that the cells were spherical, as they appeared to be). The rate of virus production per cell was calculated from the formula previously described (Rubin, 1955).

The results are presented in FIGURE 8, where relative cell volumes and rates of virus production are plotted side by side versus the number of days in culture. It is apparent that virus production and cell volume increase at approximately the same rate.

The increase in cell volume occurred almost entirely in the cytoplasm. There was no comparably marked increase in the average size or number of nuclei, which suggests that the rate of virus production is a function of total cytoplasmic activity.

The Fraction of Cells That Release Virus

The slow, constant rate of virus production in a population of normal-sized Rous sarcoma cells was quantitatively similar to the production of temperate phage in large populations of lysogenic bacteria. Before any analogy between the two systems could be presumed, it was necessary to ascertain

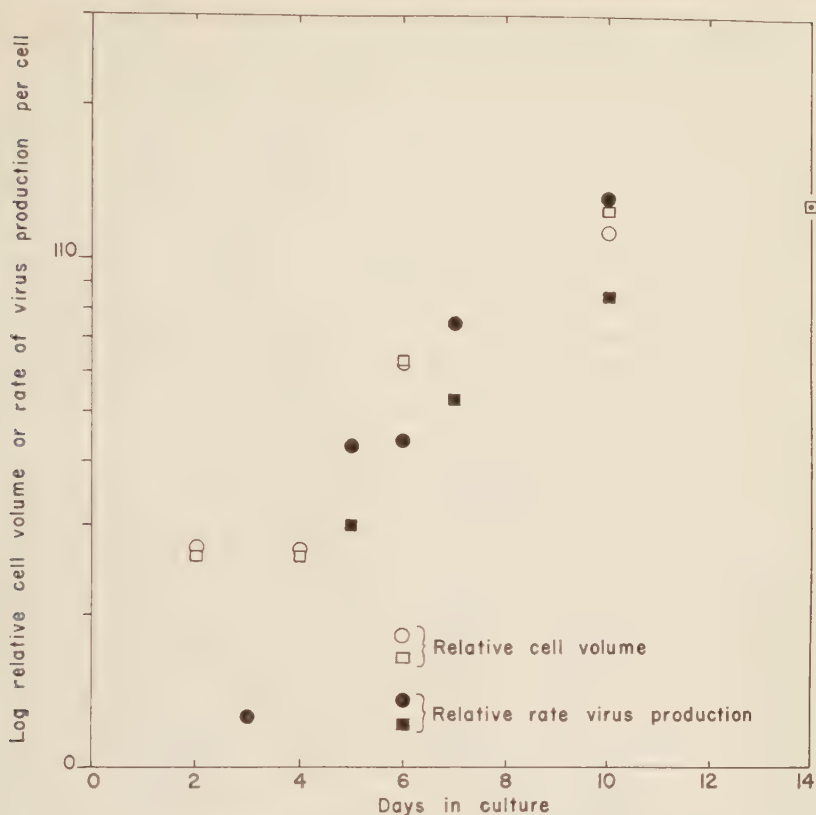


FIGURE 8. Relationship between cell size and the rate of virus production.

the characteristics of virus production at the level of the individual cell. If the two situations were truly analogous, one would expect the Rous virus to be produced in relatively large "bursts" by only a very small fraction of the cells, as is the case with lysogenic bacteria—only about 1 cell in 10,000 per cell generation yields virus in lysogenic coli (Bertani, 1951). However, there exists an alternative hypothesis that would explain the data just as well. In this model, a relatively large fraction of the cells would yield virus, but only small amounts of virus are produced by each cell. One way to distinguish between the two models is to plate a known number of the intact sarcoma cells that previously have been washed free of extracellular virus. The tumors that follow could be due, on a priori considerations, either to invasive multiplication by the implanted cells, or to the release of virus from the cells. The possibility of multiplication of the implanted cells was ruled out by an experiment in which cells were irradiated before implantation with a dose of ultraviolet light that resulted in their death within 48 hours *in vitro*, but did not decrease their ability to produce virus for about 15 hours. It was found that this level of radiation did not significantly decrease the

number of tumors produced on the CAM by the cells. This showed that the tumors were due to virus release. It was also shown that each cell could account for no more than one tumor. Therefore, each tumor represented one virus-releasing cell, and the tumor count represented the number of cells that produced virus within a certain period after implantation. After this was established, 15 experiments in which intact cells were plated at various concentrations were carried out. An average of about 1 cell in 8 yielded a tumor on the CAM, with the range running from 1 cell in 2 to 1 cell in 20.

According to these results, as compared with those obtained in the case of lysogenic bacteria, a relatively high proportion of cells released virus. It could be shown that, to contribute significantly to the number of tumors seen at 7 days, virus must be released from a cell within a day after implantation. If we assume that the sarcoma cells produce virus at the same rate on the CAM as they do *in vitro*, then calculation shows that, on the average, each cell releases only 1 to 5 t.f.u. within 24 hours.

The Plating of Intact, Enlarged Cells

Frequently, when the enlarged cells themselves were plated, on the average, several tumors per cell were produced. This was further investigated by plating only 1 or 2 of these cells per embryo. It was found that practically all the enlarged cells produced t.f.u. under these conditions. In contrast to the results with microdrops, and with the implantation of smaller cells, a few of the enlarged cells on the CAM produced virus quickly enough after implantation to infect the CAM at 20 or 30 places.

Modified Single-Cell Experiment

A second technique was used for studying, in greater detail, the production of virus by individual cells. This technique was a modification of the single-burst experiment that had its origin in bacteriophage work. From 20 to 50 Rous sarcoma cells that had been cultivated for 1 or 2 days were placed in each of 40 to 50 tubes. This number of cells was chosen after preliminary experiments showed that an average of less than 1 cell per tube would yield virus in a 6-hr. incubation period. The tubes were incubated for 6 hr., the cells were broken open by freezing and thawing, and the entire content of each tube was plated on a separate embryo. The average number of virus-producing cells per tube (m) could be calculated from the fraction of embryos that had no tumors [$p(o)$] by substituting in the Poisson equation where

$$m = -2.3 \log p(o)$$

The results showed that, under these conditions, 1 cell in 35 to 60 yielded virus in a 6-hr. period. This was somewhat less than the fraction found in plating intact cells, which is probably due to the fact that less virus is produced when the cells are damaged during dilution.

The frequency distributions of t.f.u. produced by the cells in the various experiments were also determined. These distributions were compared with a distribution that was due to the plating of small amounts of free virus on a

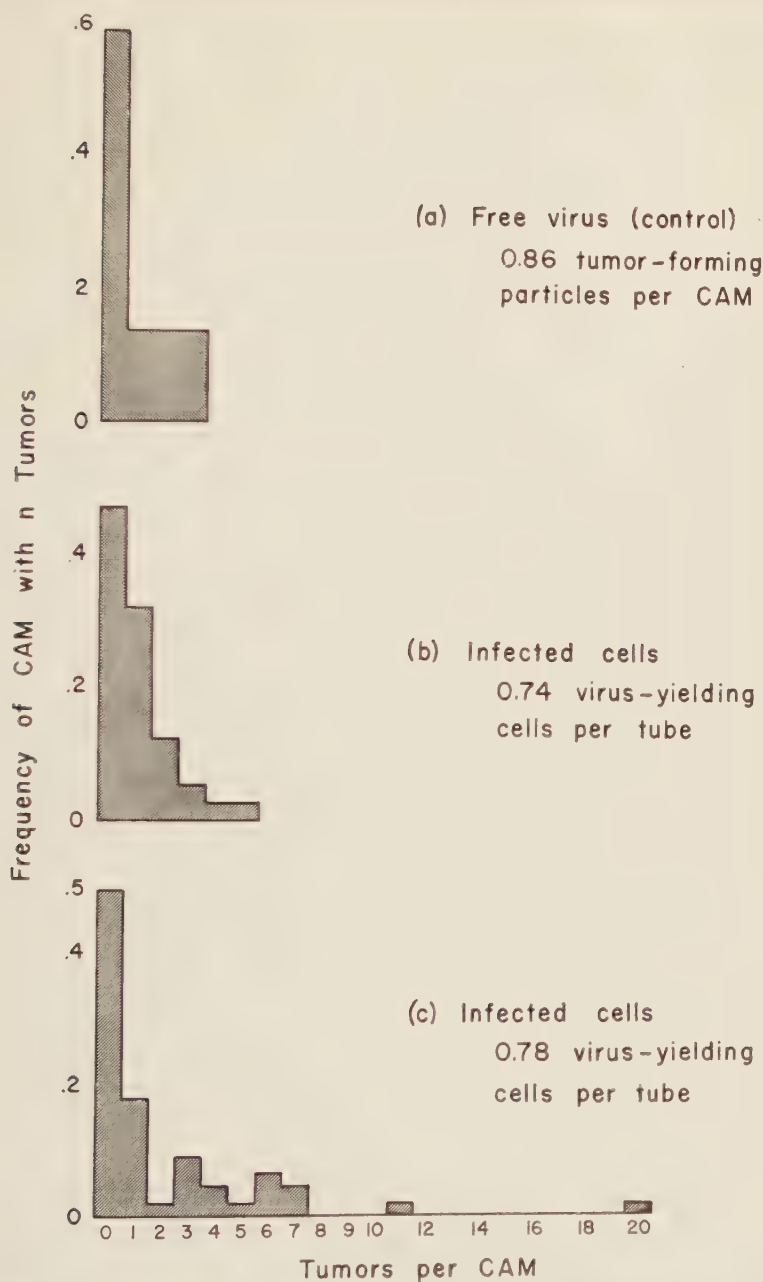


FIGURE 9. Histograms showing the frequency distributions of tumors in a modified single-burst experiment produced on the CAM of embryos due to the plating of (a) free virus, and (b) the total virus yields from individual cells.

number of embryos. The results are presented in the histograms of FIGURE 9. The distributions from plating free virus and the yields from individual cells are very similar, with only one large yield (20 t.f.u.) occurring in all of the modified single-cell experiments. Calculation shows that this yield could have been due to the presence of several virus-producing cells in the same tube. The results support the hypothesis that virus is produced in an almost random manner among a relatively high proportion of cells in the population, with the yield rarely exceeding 5 t.f.u. in a day.

Virus Production by Small Numbers of Cells in Microdrops

A study was made of the production of virus by the *enlarged* cells, at the level of the individual cell. In this case, the microdrop technique, developed by Lwoff *et al.* (1955), was used. In some respects the experiments were analogous to the single-cell experiments, but they permitted direct observation of the cells. The cells used had been cultivated *in vitro* for 10 days, and averaged 30μ in diameter. They were suspended by trypsinization, washed, and placed in a microdrop of medium on a coverslip that was under a layer of paraffin oil (Lwoff *et al.*). The system was maintained in a hood flushed with a mixture of 5 per cent CO_2 in air. The cells settled and spread on the coverslip, where they were kept under observation under a dissecting microscope. After spreading, the cells were washed by removing and replacing the medium to eliminate any extracellular virus produced up to that time, plus any cells that failed to stick to the glass. The amount of virus released at various times thereafter was determined directly by removing the entire medium surrounding the cells and inoculating it on an embryo.

A preliminary study was made of the length of time the cells could continue to produce virus in the microdrop, and of the amount of production compared to the cells in mass culture. Twelve microdrops were made with

TABLE 1
PRODUCTION OF VIRUS BY LARGE CELLS (30μ DIAMETER IN SUSPENSION)
IN MICRODROPS

Number of cells	Number of t.f.u. per drop		
	0-2.5 hr.	2.5-14.5 hr.	14.5-20 hr.
65	19	1	0
51	7	0	0
50	24	3	0
64	16	6	0
33	3	0	—
60	4	4	4
43	—	38	0
28	—	2	0
Average virus/cell.....	1/4	1/7	

TABLE 2
PRODUCTION OF VIRUS BY LARGE CELLS IN MICRODROPS

Number of cells	T.f.u. produced in 2 hr.
7	0
5	1
4	0
4	1
1	0
5	1
3	0
1	0
4	0
3	0
1	0
3	0
11	1
2	1

Rate of virus production in drops, 1 t.f.u./8 cells hr.; rate of virus production in mass culture, 1 t.f.u./1 cell hr.

33 to 65 cells per drop. The total yield was measured at $2\frac{1}{2}$, $14\frac{1}{2}$, and 20 hr. after the first fluid replacement. The results are presented in TABLE 1. For the first $2\frac{1}{2}$ hr. the cells in microdrops produced virus at about $\frac{1}{4}$ the rate of cells in mass culture. By $14\frac{1}{2}$ hr. the rate had dropped to $\frac{1}{8}$ and, by 20 hr. almost no virus was being produced.

To permit more complete observation of each cell, an experiment was carried out with 14 drops, each containing only 1 to 11 cells. Sampling was done at only one point, 2 hr. after the first washing. The results are presented in TABLE 2. The cells in this experiment produced virus at only $\frac{1}{10}$ the rate of cells in mass culture. There were no yields greater than 1 in this 2-hr. period. On an average, more than 1 in 10 of these cells yielded virus within 2 hr. It could not be determined whether the reduction in virus production relative to the mass culture was due to a decrease in either the number of cells yielding virus or the amount of virus yielded per cell, or both.

No distinctive morphologic changes occurred in any of the cells in the microdrops that contained virus, suggesting that virus release is not associated with cell death.

Discussion

The Rous sarcoma virus may cause either ectodermal or mesodermal tumors on the CAM of the developing chick embryo. The type of tumor depends, not on variation in the virus, but only on the type of cell infected. This departs from the commonly accepted concept of the rigid tissue specificity of tumor viruses. It may be argued that the ectodermal and mesodermal cells of the embryonic membrane are not as completely differentiated from one another as are adult tissues. However, there can be no question of their morphological distinctiveness.

The findings presented here show that virus is produced at a slow trickle by a high proportion of the Rous sarcoma cells when they are maintained in tissue culture. These cells remain alive, however, as measured by their ability to continue producing virus over long periods of time. These characteristics distinguish this system from lysogeny in bacteria, where only a tiny fraction of cells yield virus, and these cells are killed in the process. The distinction may be only superficial, however. Perhaps the most fundamental characteristic of lysogenic systems is not the mode of virus production, but the intimate relationship and interactions between the genetic material of the host and of the virus (see the paper by Morse, elsewhere in this monograph).

We cannot yet determine conclusively whether such a relationship exists between Rous sarcoma virus particles and their host cells. A very intimate and direct role for the virus in maintaining the cell in a malignant state is suggested, but not proved, by the unchanging quantitative relationship between the virus and the cell over many serial passages. Recent developments in tissue culture, such as clonation of cells and development of mutant cell strains, may remove this problem from the realm of pure speculation, and may permit the testing of genetic interaction between cells and tumor viruses.

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ETIOLOGY OF AVIAN LEUKOSIS*

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Leukosis, as it pertains to the chicken, is not a single disease, but a variety of neoplastic entities of distinct pathological manifestations designated collectively as the avian leukosis complex.¹ Certain of these conditions are of demonstrable viral etiology and were the first recognized members^{2, 3} of the presently known group of virus-induced animal tumors. Intensive investigations of the different forms of leukosis^{2, 4-7} have revealed a highly complicated system of seemingly interrelated diseases of well-established pathological characteristics. Despite the numerous studies on occurrence, histology, transmission, and other aspects of leukosis, until recently little has been known of the actual nature of the causative agents themselves. A beginning has now been made in the experimental resolution of some of these etiological enigmas by the isolation and direct study of the physical, chemical, and biological properties of viruses of representative forms of the complex. The findings summarized here not only afford elements of clarification as regards etiological problems of leukosis; they also reveal unequivocal evidence of a relationship between leukosis and the avian malignancies classified as the chicken sarcomas.⁸

The Problem

The diseases of avian leukosis, in the rather restricted and conservative definition of Jungherr,⁶ "are such which are primarily characterized by autonomous proliferation of essential blood-forming cells, and are, as a rule, due to oncogenic viruses." The principal forms of the complex are shown in FIGURE 1. In one category are the neoplastic states exemplified by the commonly occurring visceral lymphomatosis characterized by collections in the liver, lung, and other viscera of primitive cells of lymphoid origin—growths that, in effect, constitute disseminated lymphosarcomatosis (see the paper by Burmester elsewhere in this monograph). Other frequently occurring lymphoid states are neurolymphomatosis and ocular lymphomatosis, in which lymphoid accumulations are associated with nerve trunks and the eye, respectively. A distinct disease often found with any or all of the forms of lymphomatosis and classified with them is osteopetrosis, an involvement of the periosteum resulting in overgrowth of bone. In contrast with lymphomatosis are the leukemias: myeloblastosis,⁹ in which hordes of primitive cells of myeloid origin reach the circulation, and the erythroid disease,^{5, 10} manifested by the presence (also in the circulation) of large numbers of erythro-

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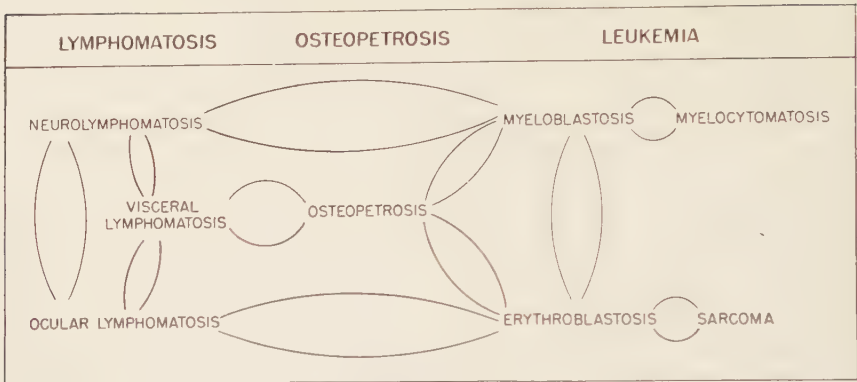


FIGURE 1. The major conditions of the avian leukosis complex. The connecting curves indicate, in part, the diversity of combinations occurring naturally, or transmissible by experimental means.

blasts. Lymphomatosis rarely causes changes in the circulating cellular elements.⁴ A less frequently encountered disease is myelocytomatosis. Several strains of erythroblastosis have been complicated by concurrent sarcoma.^{5, 11}

The earlier studies of these chicken diseases have resulted in recognition of the facts that (1) each condition is a distinct pathological entity that may, and does, occur individually; (2) any two or more may be found together in nature; (3) various mixtures can be propagated by cell transplants or by virus transmission with filtered material; and, finally and most inexplicably, (4) some of the diseases, particularly the leukemias, may be derived from birds with some form of leukosis, but showing no evidence of the derivative condition.

Knowledge of the etiological aspects of the various conditions has been limited. It has been repeatedly demonstrated that myeloblastosis and erythroblastosis are transmissible by filtered extracts or fluids. Lymphomatosis and the associated condition, osteopetrosis, are likewise transmissible by this means,⁴ but such conditions as neurolymphomatosis, ocular lymphomatosis, and osteopetrosis, although apparently of viral origin, have not been transmitted in serial passage with cell-free materials. They do occur, however, as mentioned before, in filtrate transfer of visceral lymphomatosis.

With the previously well-established viral etiology and the extensive background of the pathology of the diseases, we undertook systematic studies with the initial purpose of physically isolating and identifying one or more of the associated viruses. Although earlier efforts of this sort had been made,^{12, 13} the results were limited by conditions related principally to virus concentration and variation from one host to another and recognized in the present work only after considerable experience. The first studies in this laboratory were made with visceral lymphomatosis (RPL 12 strain¹) and, due to difficulty in separating virus from cell extracts, these were no more rewarding than those made by other investigators. At the suggestion of

E. P. Johnson, the work was resumed later¹⁴ with a strain of myeloblastosis* which he provided. This material, derived originally¹⁵ from two cases of neurolymphomatosis and associated from time to time¹⁶ not only with lymphomatosis of different forms but with erythroblastosis, has appeared to continue as a pure strain, *by passage with filtered material alone*, for seven years. Through a series of fortunate circumstances, as well as through the singular properties of the myeloblastosis virus and its occurrence in very high concentration in blood plasma, it has been possible to isolate the agent and to characterize it by its physical and chemical properties¹⁷⁻²¹ and its biological characters, with respect both to problems of host response^{22, 23} and to antigenic behavior.²⁴ With the knowledge afforded by myeloblastosis, the investigations could be extended to the agent of erythroblastosis,²⁵⁻²⁹ which turned out to be a much more difficult problem. Erythroblastosis, derived by Engelbreth-Holm⁵ from a case of myeloid leukosis, likewise has behaved as a pure strain.²⁵ The experience with myeloblastosis also stimulated further studies of visceral lymphomatosis, which resulted in the demonstration³⁰ of plasma particles characteristic of the disease and in immunological studies carried out with it in collaboration with B. R. Burmester of the Regional Poultry Research Laboratory, East Lansing, Mich.

It is my aim here to cite briefly the results of experiments that give the basis for the comparison and, as it proved, the differentiation and interrelationships of the agents of myeloblastosis, erythroblastosis, and visceral lymphomatosis, RPL 12 strain⁴ (indicated hereafter as lymphomatosis). As the result of recent preliminary studies with W. Ray Bryan with the Rous sarcoma, and of the earlier findings of Andrewes,³¹⁻³³ extension of interpretations to the area of the chicken sarcomas has been possible.

Physical Attributes

The morphology of particles characteristically associated with myeloblastosis, erythroblastosis, and lymphomatosis is illustrated in the electron micrographs of FIGURE 2. In myeloblastosis, spheroidal particles such as those of FIGURE 2a^{17, 24} occur in the plasma of diseased birds at levels as high as 2×10^{12} per ml. In consequence of this concentration, which represents about 1.5 mg. of hydrated virus per ml., the particles are available in sufficient amounts for quantitative studies by diverse procedures. Identification of the particles with the infectious entity of the disease has been thoroughly established by direct particle counts, analytical and fractional ultracentrifugation,¹⁷ fractional electrophoresis,^{17, 21} and, finally, by specific precipitation with antiviral chicken immune serums.²⁴ Problems of a different magnitude were encountered with the particles in the plasma of birds with erythroblastosis shown in FIGURE 2b. Such particles occur in far fewer numbers,²⁹ approximately 10^{10} per ml., and identification has been limited thus far, but specifically established,^{25, 27} nevertheless, by precipitative procedures with antierythroblastosis chicken immune serum. The

* Because of the previously unknown etiologic relationship between myeloblastosis and erythroblastosis, the designation "erythromyeloblastosis" leukosis was employed in earlier publications. The agent is now regarded as the virus of myeloblastosis.

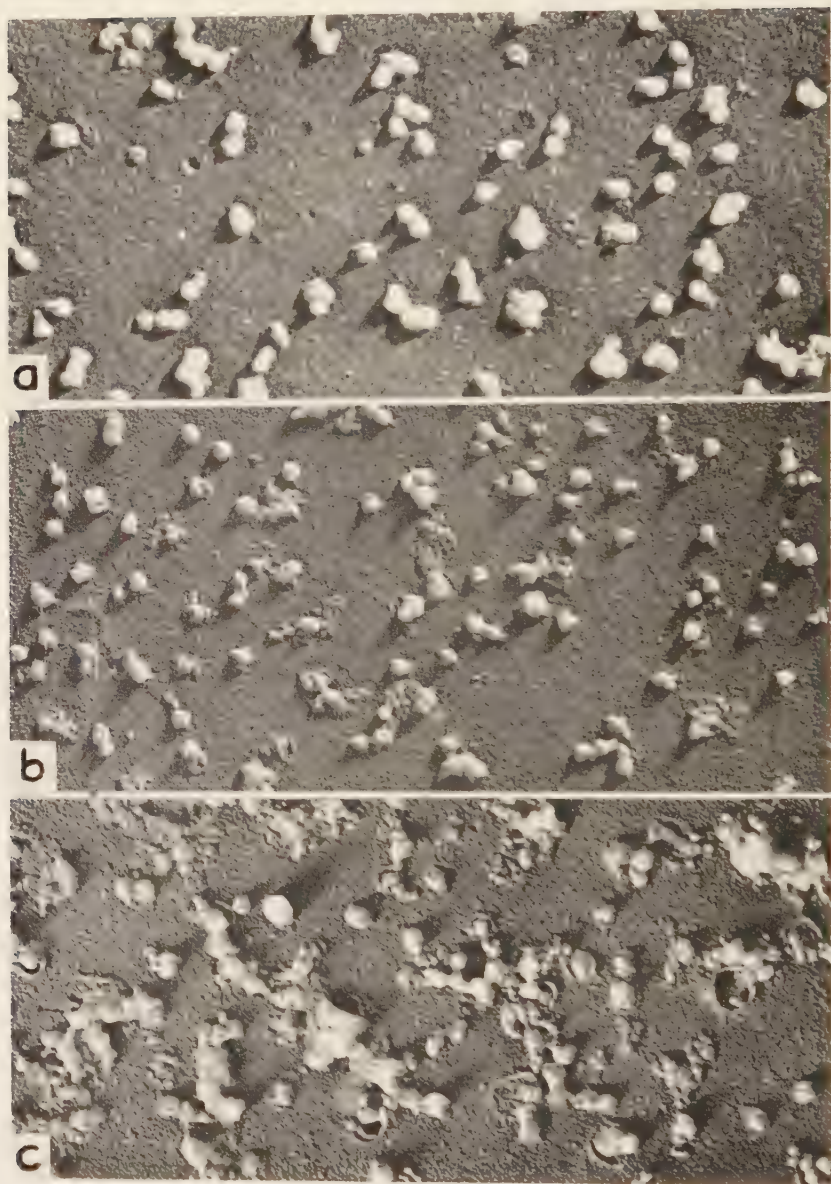


FIGURE 2. (a) Virus of avian myeloblastosis in a concentrate of the plasma of a bird with the disease; (b) virus of avian erythroblastosis in a concentrate from plasma; (c) characteristic particles observed in a concentrate from a bird with visceral lymphomatosis (RPL strain 12). $\times 24,000$.

small number of virus particles has precluded comprehensive experimentation by physical methods. The particles of FIGURE 2c were observed³⁰ in the plasma from a chicken with lymphomatosis. Only one plasma of about twenty-five specimens examined has shown particles in high concentration, but they were observed in smaller numbers in three other samples. Identification with the virus of this disease has not been established, but it is likely that it represents the infectious agent.

It is characteristic of the three forms of leukosis that the number of particles varies greatly in the individual birds,^{19, 20, 34, 35} a circumstance hindering experimentation if no means is available for selecting good plasmas. Some plasmas from birds dying of myeloblastosis contain few particles, less than 10^9 per ml. (the limits of the physical and chemical methods of determination), and other plasmas from definitely diseased chicks do not contain virus demonstrable by infectivity tests.^{36, 37} Likewise, variation (the extent of which cannot be judged) occurs in erythroblastosis, not many plasmas contain as many as 10^{10} particles per ml. There is no basis for speculation on the limits of variation in lymphomatosis, although one plasma³⁰ contained approximately 10^{11} or more particles and, in most, no significantly identifiable particles could be detected.

In morphology, as shown by electron microscopy, there are no distinguishing differences between the particles, although there is some evidence that those of myeloblastosis²⁰ are slightly larger (120 $\mu\mu$ diameter in electron micrographs) than those²⁹ of erythroblastosis (about 100 $\mu\mu$ in diameter). Studies using ultracentrifugation and still in progress³⁸ indicate that the sedimentation rate of myeloblastosis virus is slightly greater than that of erythroblastosis, a result not in disagreement with the findings by electron microscopy.

A consideration of the physical findings reveals apparent similarities between the particles, but provides no bases for judgment of close relationship of properties, or for distinguishing between the respective particle populations.

Host Response

Numerous studies have been made of the interaction of the viruses and the chicken host that results in the induction of the individual diseases. The patterns of host response to the agents of myeloblastosis^{22, 36, 39} and erythroblastosis²⁸ are closely similar in principle, but very different when observed in terms of the magnitude or the intensity of the reactions. In both instances the level of population response was influenced greatly by genetic factors.^{28, 40} One strain of chickens, Line 15 White Leghorns, long inbred^{41, 42} for high susceptibility to lymphomatosis, were similarly of relatively low resistance to the leukemia viruses. Other breeds and strains of chickens were strongly resistant to infection with either agent. The response of the Line 15 chicks, the most susceptible studied thus far, was characterized,³⁶ nevertheless, by very high median population resistance and an extremely broad variation in individual susceptibility. In myeloblastosis, for example, the median effective dose, ID_{50} , of virus was about 26 million

particles. In reckoning with the individual bird, however, it was found that about 6000 particles sufficed to cause disease in 5 per cent of the birds, while about 145 billion were required to produce an incidence of 95 per cent positives, a difference of 24 millionfold. Analogous studies of the same quantitative nature have not been made in erythroblastosis, but it is evident that the range of individual variation is not greatly different from that in myeloblastosis.

A very great difference was seen, however, in median population resistance to the two agents. The erythroblastosis virus was relatively highly "virulent," producing a given level of disease with numbers of particles one hundredfold to one thousandfold less than those needed in myeloblastosis. A marked difference was seen^{22, 28} also in the rate both of the induction of the diseases and the death of the host. In erythroblastosis, the onset could be recognized²⁸ within 48 hours, and death could occur as soon as 6 days, with a mean of 9 days, after inoculation. A longer period was observed in myeloblastosis; primitive cells were seen in 9 days, and deaths occurred as early as 12 days, with an average of 17 days after inoculation of the virus. Still less intense reactions were seen¹⁻³³ in lymphomatosis in which the average survival of birds after receiving virus was in the range of 100 days. Although quantitative studies involving particle counts have not been made with lymphomatosis, it has been obvious that the long incubation period in the disease is characteristic of this specific host-virus interaction and is by no means attributable to the level of the virus particle number.

Another important difference in host responses to the three agents is concerned with the age of the bird at the time of inoculation. The greatest susceptibility to the virus of myeloblastosis was seen with 3-day-old chicks, and resistance increased rapidly with age.²⁵ A like relationship was seen in lymphomatosis in which susceptibility was inversely related to age.⁴ In striking contrast, susceptibility to erythroblastosis²⁶ was far less at the 3-day age than at 77 days. With all three viruses, the most effective route of inoculation was directly into the blood stream.

The results of such biological experiments alone indicate unequivocal differences in the apparent properties of the agents in their effects on the same strain of hosts. It could be said, however, that the differences are not necessarily related wholly to the activity of the agents themselves. In these processes, three distinct types of cells are involved. It is conceivable that a single virus might be responsible, infection of a given cell depending largely not on virus specificity, but on the state of receptivity of the cell at the time of inoculation. Such a theory has been advanced⁵ to explain frequent mixtures of the leukemias. It must be conceded that the results cited above do not exclude the possibility that the apparent differences between the myeloblastosis and erythroblastosis viruses with respect to incubation period and age influence might be due for the most part to properties inherent in the cell affected and not to those of the virus. A more likely interpretation is that based on the concept that myeloblastosis, erythroblastosis, and lymphomatosis are distinct disease entities effected by agents specific to the stem cell involved, and that the resulting condition is merely the vector of

the unique reactions between the specific virus and the specific host cell. All influences such as those of age, genetic constitution, route of inoculation, and others thus may bear on the phenomena of disease production only through modification of a specific cell-virus reaction and not by induction of some other reaction.

Enzymatic Activity

That the virus of myeloblastosis differs from that of erythroblastosis is quickly demonstrable by enzyme studies. With the agent of myeloblastosis there is associated a pronounced activity to dephosphorylate adenosine triphosphate.^{18, 19, 35} Correlation of enzyme activity with the particles and with the virus has been firmly grounded by many quantitative experiments^{17, 19, 21} involving analytical and fractional ultracentrifugation, particle counts, and electrophoresis, and by serologic procedures,²⁴ including specific precipitation with homologous immune serums. There is no question that the enzyme accompanies the virus. Whether it is an intrinsic constituent of the agent or is adsorbed on it while inside the host cell⁵ is not pertinent to the present discussion.

In striking contrast to this property of the virus of myeloblastosis is the extremely low level or the essentially complete lack of such activity^{28, 38} on the part of the agent of erythroblastosis. No plasma from birds with erythroblastosis has ever given a reaction with the microadenosine triphosphatase (ATPase) test,^{34, 45} and quantitative studies³⁸ based on particle counts have confirmed the findings with the micro test. Investigations of the reactions with plasma of measured particle content have given values of specific particle activity averaging 10 per cent of the property of the myeloblastosis virus. When the particles were washed by repeated sedimentation there was only a suggestion of enzyme activity. This profound difference is of interest not only as a distinguishing characteristic, but much more so in view of the close biological relationships between the agents demonstrated by other means.

Antigenic Properties

The quantitative physical and chemical studies have provided an excellent foundation for work with the agents by immunologic and serologic procedures. Initial investigations were made with myeloblastosis,²¹ which could be obtained in suitable amounts for the immunization of chickens and other species. This was accomplished in chickens with formalin-inactivated virus followed by large doses of active virus in highly active plasmas selected by the micro ATPase test. Moreover, even fully active viruses were used throughout by employing adult chickens, most of which were resistant to infection. The antibodies thus produced strongly neutralized and precipitated the agent. Rabbits injected with purified concentrates of virus yielded immune serum capable of neutralizing virus and fixing complement, but which, on macroscopic inspection, demonstrated no evidence of precipitation.

The results of the studies with these sera demonstrated that the virus contained an antigen specific to the agent that was active in the homologous host, the chicken. This same antigen was active, likewise, in the production in the rabbit of analogous antibodies that were effective in neutralizing the agent. A singular phenomenon came to light when it was found that the rabbit antibodies fixed complement not only with virus concentrates, but with suspensions of normal chicken tissue. This observation stimulated further studies in which it was found that antibodies resulting in the rabbit from the injection of normal chicken tissue or normal chicken serum also fixed complement with virus and, in addition, were capable of *neutralizing virus infectivity*. Revealed by an extension of the studies, using Forssman antigen, a component of normal chicken tissue, was the phenomenon of virus neutralization by Forssman antibody derived from rabbits injected with guinea pig kidney tissue. These observations have been interpreted as suggesting that the virus of myeloblastosis contains (1) a specific component peculiar to the virus and active in the homologous host; (2) a second antigen acting immunologically as normal chick tissue; and (3) another material of the character of Forssman antigen. It seems apparent that these antigens must be either intrinsic constituents of the infectious moiety of the virus particle, or closely bound to it.

Similar experiments with erythroblastosis^{25, 27} have been limited by the small amounts of virus available. Nevertheless, formalin-inactivated concentrates of virus obtained from a large volume (more than 2 l.) of plasma from about 200 chickens with the disease have been used for vaccination of, thus far, 8 normal chickens which, in the process, never showed evidence of the disease. The antierythroblastosis chicken immune serum neutralized the active virus to a highly effective degree, as did the myeloblastosis immune serum with its homologous virus. Sufficient erythroblastosis virus has not been available for the injection of rabbits, but studies with the antinormal chicken-tissue or chicken-serum rabbit immune serums used with the virus of myeloblastosis also neutralized the agent of erythroblastosis. This provided evidence of antigenic similarity between the two leukemia viruses with respect to the presence of normal chick-tissue component in association with the infectious portion of the particles. A difference was observed between myeloblastosis and erythroblastosis, however, with Forssman antibody. There was *not the slightest evidence of neutralization* of the erythroblastosis virus (contrary to the finding with myeloblastosis) by immune serum induced in the rabbit with guinea pig kidney tissue. Here, then, exists a clear and definitive serological distinction between erythroblastosis and myeloblastosis.

In lymphomatosis of a long host-survival period, neutralizing antibodies occur in diseased birds and may be produced or accentuated by the administration of large doses of virus.^{16, 17} Although the existence of these antibodies has been clearly demonstrated, as will be shown, the experiments have not been extended to precipitation. Investigations with antinormal chick-tissue and chicken-serum rabbit immune serum and with Forssman antibodies are now under way in Burmester's laboratory.

Serologic Interrelationships in Leukosis

Access to these antiviral immune bodies has provided the means for very enlightening cross-serologic experiments (illustrated in a portion of FIGURE 3). Antimyeloblastosis chicken immune serum has neutralized,²⁵ apparently with equal effectiveness, both myeloblastosis and erythroblastosis viruses. The opposite combination of antierythroblastosis immune serum with the respective agents resulted in like cross neutralization. Erythroblastosis virus also was strongly neutralized by the antimyeloblastosis virus immune serum from the rabbit. In these experiments there was no evidence of qualitative or quantitative differences in specific antigenicity. With Burmester, it was observed that antimyeloblastosis immune serum neutralized the RPL 12 strain of lymphomatosis virus; the converse reaction was found between antilymphomatosis immune serum and myeloblastosis virus.

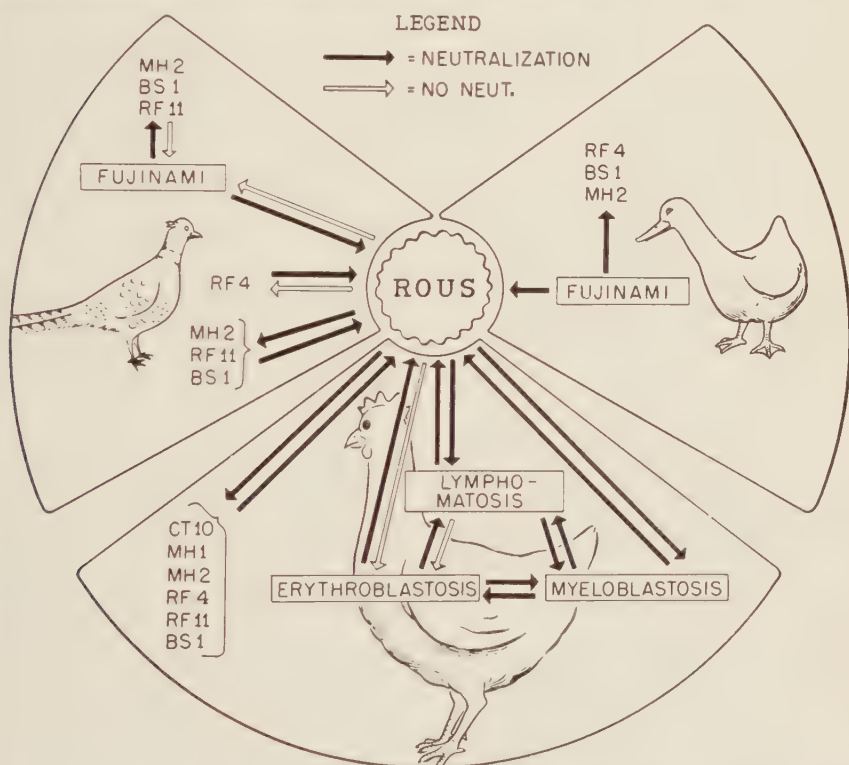


FIGURE 3. Immunological interrelationships between the chicken tumor viruses, between the various forms of avian leukosis indicated, and between the Rous sarcoma virus and the avian leukosis viruses. The solid arrows indicate neutralization of the virus at the point by immune serum to the virus at the base. The open arrows signify no neutralization in tests already made. The immune sera were derived in the bird indicated. Location of the Rous virus as the apparent pivot is an artifact due to the larger numbers of experiments done with the agent. Central significance of the Rous virus is not implied.

These reactions were not obviously of the same order as those between the leukemia viruses themselves. Tests have revealed a major difference with erythroblastosis, since this agent, even in limiting high dilution (that is, the least amount of virus compatible with interpretable infectivity tests), was not neutralized by antilymphomatosis immune serum capable of neutralizing either lymphomatosis or myeloblastosis virus. The experiment with anti-erythroblastosis immune serum and lymphomatosis virus is still to be made.

Correlation of the results thus far achieved yields the interpretation that the viruses of myeloblastosis and erythroblastosis are closely related serologically. Furthermore, because of the interrelationship between the leukemias and that between myeloblastosis and lymphomatosis, it is evident that the three agents are all of related antigenic structure.

Precipitin Reactions

Although there was no apparent difference between myeloblastosis and erythroblastosis in the neutralization test with chicken immune serum, a definite distinction was evident²⁵ in cross-precipitin reactions studied by electron micrography. Antimyeloblastosis immune serum from the chicken quantitatively precipitated the homologous virus but, under the same conditions in the same experiment, produced only partial precipitation of the virus of erythroblastosis. Antierythroblastosis chicken immune serum has precipitated *only partially* the virus of erythroblastosis, and has had little if any precipitative activity against the same preparation of myeloblastosis virus that was completely precipitated by the homologous immune serum. While there are many variables governing the quantitative aspects of serologic precipitation, it seems clear that, under the conditions of the experiments, the two antigens were not identical in their participation in the heterologous reactions.

Avian Leukosis and the Avian Sarcomas

There have been many speculations regarding the possible relationships between the various forms of avian leukosis and the analogous neoplasms of the chicken—the sarcomas—as exemplified by the Rous tumor. Much work has been done, particularly by Andrewes,⁵¹⁻⁵³ in an attempt to resolve the relationships among the virus-induced chicken tumors themselves. The results by Andrewes are illustrated in the diagram FIGURE 3. Here it is indicated that neutralization experiments carried out in the chicken have shown definite, although varying, cross reactions between the viruses of the Rous, CT 10, MH 1, MH 2, RF 4, RF 11, and BS 1 chicken tumors. All are spindle-cell or fibrosarcomas except MH 2, which is an endothelioma. With few exceptions, like relations were found when the immune serums were produced in the pheasant. A major difference was the observation that antibodies to the RF 4 agent from the pheasant were active against the RF 4 virus and against the Rous agent, but anti-Rous virus immune serum did not neutralize the RF 4 virus. Cross relations were seen between the Rous, MH 2, RF 11, and BS 1 viruses with the serums from the pheasant.

Another growth, the Fujinami myxosarcoma of the duck, produced in this

bird an immune serum that neutralized the Rous, BS 1, RF 4, and MH 2 viruses, but the reaction proceeded in only one direction. The same relationship occurred with anti-Fujinami immune serum from the pheasant. In no case, however, was neutralization of the Fujinami virus observed with the antibodies from the other sarcoma viruses, whether produced in the chicken, the pheasant, or the duck. These findings obviously led Andrewes to the concept that the various chicken tumors, and the Fujinami tumor as well—all of those examined for this purpose—were interrelated etiologically and were the result of various forms of a family of biologically related agents.

Through collaborative studies on myeloblastosis and erythroblastosis at Duke University, on lymphomatosis by Burmester, and on the Rous sarcoma by W. Ray Bryan, an opportunity has been afforded for direct tests for an interrelationship between the Rous virus and the leukosis viruses. In work still under way it has been found that anti-Rous virus immune serum from the chicken neutralizes the agents of both lymphomatosis and myeloblastosis. The converse reaction with antimyeloblastosis and antilymphoblastosis immune serum has resulted in the neutralization of the Rous virus. Anti-erythroblastosis immune serum has neutralized the Rous virus, but there has been no evidence of neutralization of erythroblastosis by anti-Rous virus immune serum.

The results of these experiments, although still in the preliminary stages, warrant the interpretation of a definite etiological interrelationship between the various forms of leukosis and the chicken tumors investigated. It appears that the large number or family of virus-induced neoplastic conditions of the chicken is the result of the activity of various members of a correspondingly large family of closely related, although distinct, agents.

Summary

The observations described here have provided evidence of a kinship between the viruses of the avian leukemias, myeloblastosis, and erythroblastosis. In addition, the properties of the agents in their relationship to the host, in the presence or absence of adenosinetriphosphatase activity, and in their reactions with homologous and heterologous immune sera have clearly indicated that the agents are separate and distinct biological entities. This problem has now been clearly resolved with respect to the leukemias.

Similar studies by serologic procedures alone have revealed relationships and distinctions between the agents of the leukemias and the virus of lymphomatosis.⁴ Thus, the three agents definitely appear to constitute members of a family of viruses parasitizing, separately and specifically, the respective stem cells of the hematopoietic system. Furthermore, there is now definite experimental evidence on which to base the conclusion that these viruses of leukosis are but one branch of a far larger system of related agents responsible separately not only for leukosis, but for the numerous recognized virus-induced avian sarcomas that have been studied from this point of view.

The findings provide the basis for further explanations in this area, which is one of most fundamental significance in the development of established

concepts bearing on the occurrence and behavior of the chicken neoplasms and of other virus-induced conditions existing in complex states in other animals. Within the sphere of the chicken tumors, recognition of the virus of lymphomatosis as a member of the family group is of special importance. Lymphomatosis is the only form of avian malignancy thus far known (see Burmester¹ and elsewhere in this volume) to be *contagious* in nature. It is not overly remote to speculate that the agent of lymphomatosis may constitute the transmissible stem virus that, on passage in the chicken population may, by variation, mutation, and selection, provide the source of specific transformed entities that, by virtue of restricted cell-virus relationships, lose contact with the mechanisms responsible for spread of lymphomatosis. Thus it seems entirely likely that transmission of lymphomatosis by contagion is but the natural means for the maintenance in the chicken population, not only of the various forms of leukosis, but of the virus-induced chicken sarcomas, as well.

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ROUTES OF NATURAL INFECTION IN AVIAN LYMPHOMATOSIS

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Introduction

There are three recognized forms of avian lymphomatosis—ocular, neural, and visceral.¹ These terms are based on the type and location of the principal pathological lesions without respect to their etiology. A combination of two, and occasionally all three, of these forms may occur in the same flock of chickens, or even in the same individual. A further segregation has been made by certain English workers,²⁻³ who have classified lymphoid tumors of the viscera (visceral lymphomatosis) into two types: those that are associated with neural lymphomatosis and are primarily lymphocytic, classified as the visceral form of neural lymphomatosis (fowl paralysis); and those composed primarily of lymphoblasts, classified as lymphoid leukosis (lymphoblastomas and lymphosarcomas). Some investigators have claimed that the three forms of lymphomatosis, as well as other leukotic diseases of chickens, are caused by a single agent; however, recent information indicates that all forms of lymphomatosis, osteopetrosis, the leukemic diseases, myeloblastosis and erythroblastosis, and other transmissible tumors are all caused by a family of viruses, each having distinctive features, but with certain characteristics in common.

Conditions similar to those known today as neural and visceral lymphomatosis were first reported at about the turn of the century.⁴ During the first two to three decades of this century the occurrence of neural lymphomatosis increased to such an extent, apparently spreading across the country from east to west, that it became a particular concern to research workers in animal diseases. The ocular form also made its appearance and, by 1930, the three forms of lymphomatosis had become a definite economic problem on most farms in practically every section of the United States. The general impression prevails that neural lymphomatosis caused its highest mortality prior to 1930. The occurrence of the visceral form increased more slowly, but it has now become more prevalent than the neural form, and is responsible for two thirds to three fourths of all mortality from the disease. Similar observations on the spread of these diseases were noted in England,^{5, 6} Australia,⁷ and Japan.⁸

The upswing in the occurrence of lymphomatosis generally coincided with the development of modern methods of incubation and other practices of intensive poultry keeping. Whether there is more than a coincidental relationship is open to conjecture.

Of the various infectious neoplasms of the chicken, only visceral lymphomatosis has become prevalent under commercial rearing conditions; this

creates a great problem for the poultryman. It is apparent that visceral lymphomatosis is contagious; on the other hand, erythroblastosis, myeloblastosis, osteopetrosis, the fibrosarcomas, the myxosarcomas, the hemangio-endotheliomas, and others are highly infectious, but are not contagious in the usual sense.

Observations on the natural occurrence and spread of lymphomatosis suggest that natural infection may occur (1) through the egg, from infected parents to the offspring; (2) by contact with air, feed, water, equipment, or caretakers that have become contaminated with infected secretions, excretions, or droplet nuclei; or (3) by direct contact of infected with noninfected birds.

Egg Transmission

Doyle,⁹ in 1928, was the first to suggest that neural lymphomatosis was transmitted by the egg; subsequently the concept was broadened to include visceral lymphomatosis. Indirect evidence in support of this mode of transmission was soon reported by several investigators. Some investigators^{7, 8, 10-13} introduced hatching eggs only into isolated areas where chickens had not previously been reared, and they obtained a high incidence of the disease. Results of incubator exposure experiments¹⁴ and other transmission studies¹⁵ also indicated transmission by the egg. In contrast to this view, egg transmission was minimized¹⁶ and, subsequently, evidence indicating that lymphomatosis was not transmitted through the egg was presented.¹⁷

Direct evidence that the agent of visceral lymphomatosis (VL) is present in the egg was only recently obtained.¹⁸ Infectivity tests of embryo liver suspensions demonstrated the presence of the VL virus in the eggs of certain dams and its absence in the eggs of others. In subsequent experiments,¹⁹ 22 hens were selected at random from an infected flock of White Leghorn chickens. Of these, 17 were from 2 susceptible inbred lines, and 5 were from a line selected for resistance to lymphomatosis. Birds of all lines were reared together, thus being exposed to the same environment during the same period of time. The presence of the virus in 15-day embryos from 4 to 5 embryonating eggs of each dam was determined by inoculating groups of 40- to 50-day-old chicks of a susceptible line, free from prior infection, and observing the occurrence of lymphomatosis. It was found that 16 of the 17 hens from the 2 susceptible inbred lines laid eggs that, when incubated up to 15 days, contained enough virus to produce a significant incidence of VL in inoculated chickens. In contrast, only 1 of 5 hens from the resistant line showed evidence of shedding the virus into her eggs. These tests were made while the hens were about 1 year of age, and on eggs laid during March. Similar tests were conducted on eggs laid 3 months later and on others laid 6 months later. Evidence of some variation in shedding of the virus by individual hens was obtained, but a generally high level of virus in eggs was evident for hens of the 2 susceptible lines. However, tests made on eggs laid when the same hens were 2 years and 3 years of age gave transmission results indicative of a much lower level of virus.

Progeny of the hens of the susceptible lines were hatched from eggs laid when the dams were about 1 year of age. Infectivity tests conducted on the eggs laid by the progeny when they were 8 to 9 months of age showed that 9 of 14 progeny had shed significant virus into their eggs. Subsequent tests of eggs laid when the progeny were about 1 year and about 2 years of age gave much lower rates of transmission.

It should be emphasized that all hens were apparently in normal health at the time the eggs for the infectivity tests were laid. Furthermore, during the year following the laying of eggs for the first test of the original 22 hens, 8 hens died of various causes. The death of only 3 of these was attributed to lymphomatosis; one of them died within 2 weeks and the other 2 almost a year after the eggs for the infectivity tests were laid. Eggs infected naturally or by inoculation hatch well, and the chicks have high livability.²⁰

These results demonstrate quite conclusively that the virus of VL had been present in eggs while the hens, the embryos, and the chicks appeared normal and in good health. The only source of the virus resided in the hen that laid the eggs. Since it has been shown²⁰ that virus in embryonating eggs propagates during incubation, the response obtained in chicks inoculated with 15-day embryonic liver was the result of virus deposited and then propagated during 15 days of embryonic development.

It would appear that the original amount deposited by the hen is of importance in influencing the amount of virus detectable at the 15th day. Data^{19, 21} show that genetic constitution and age are important factors influencing the deposition of virus in eggs of hens of an infected flock. Young hens of a line selected for susceptibility to lymphomatosis were more apt to shed significant amounts of virus into their eggs than old hens or those of resistant lines.

Although both neural and visceral lymphomatosis occurred in the flock with the hens that supplied the eggs for infectivity test, only VL occurred to any significant extent in the test chicks inoculated, thus adding further to a large accumulation of data²²⁻²⁷ indicating that the causative agent of neural lymphomatosis is different from that causing visceral lymphomatosis. The lack of evidence for the presence of the causative agent of neural lymphomatosis in eggs of hens from the infected flock is in agreement with the results obtained by investigators at Cornell University,^{16, 17, 28, 29} Ithaca, N. Y. These workers reported no evidence for egg transmission of "leukosis." A breakdown of their diagnoses at necropsy shows that over three fourths of all cases of lymphomatosis were of the neural form.³⁰ Hence, interpretations and conclusions based on such data should be confined to neural lymphomatosis.²⁴ Contrary to the first suggestion of egg transmission and early confirmatory reports, based largely on neural lymphomatosis, the more recent data^{16-19, 21, 28-30} are negative with respect to egg transmission of the neural form of the disease.

Although it has now been shown beyond question that hens shed the VL virus in eggs, and that their embryos become infected and provide a medium for the multiplication of the virus, one must assess the significance of this in relationship to the natural transmission of the disease. Does such infec-

tion influence the occurrence of VL in the progeny? Is it the source of infection for penmates of the progeny?

A study was made^{19, 31} of the occurrence of VL in 2 sets of progenies of hens classified with respect to shedding virus into their eggs. Both sets of progeny were obtained from dams at an age when some (12) were classified as heavy shedders, others (5) as light shedders, and still others (5) as non-shedders. One set of progenies of about 10 birds per dam were hatched and reared together in regular incubators and brooder rooms, allowing contact between families, with caretakers and the usual equipment of the incubator and brooder unit. The second set of about 15 birds per dam was incubated and hatched in special family isolation units and then brooded to 3 months of age in isolation cubicles, after which the birds were moved to regular pens and held until 500 days of age.

The incidence of VL in the first set of progenies was generally higher than that in progenies reared in isolation. Both sets of progenies gave similar results, however, in that there was no apparent relationship between the amount of virus in the embryos of specific dams and the occurrence of VL in the progenies of the same dams. Thus it was found^{19, 31} that many hens classified as heavy shedders produced progenies, none of which developed lymphomatosis during a 500-day period, and some from dams classified as non-shedders developed a few cases of lymphomatosis.

It would appear, therefore, that the presence or absence of the VL virus in embryos was not the most important factor in determining whether such chickens, when hatched, would later develop lymphomatosis.

It has been suggested that the unexpectedly low incidence of VL in the progeny of shedder dams was due, in part, to the passage of maternal antibodies to the chick via the egg, as occurs in several other chicken diseases. An adequate basis for this suggestion was provided by the demonstration that, when chickens were given repeated injections of the VL virus, they developed neutralizing antibodies.³² The progeny obtained after the injection were much more resistant to challenge inoculation than were the progeny of the same hens obtained before the immunizing injection.^{27, 33} Neutralizing antibodies have been found also in the serum of day-old chicks hatched from eggs of hens that had received immunizing injections and from hens that had been naturally infected and had shown previous evidence of shedding the VL virus into their eggs.³⁴

Contact Transmission

The importance of the egg-transmitted virus becomes clear when we consider several experiments on contact transmission, in which baby chicks of an infected flock were used as the sole source of infection in exposing chicks from a noninfected source.

Numerous investigators^{6, 11, 13, 14, 26, 31, 35, 36} have shown that chicks of a relatively disease-free source, when reared in the same brooder units with other chicks from an infected flock, develop a high incidence of lymphomatosis that is usually greater than the incidence in the progeny of the infected flock. Age at exposure appears to be important, since it has been

found^{14, 26, 37-39} that, in any one experiment the younger the birds at time of exposure, the greater the incidence of disease. Furthermore, the virus of VL was found to be present in debris of an incubator that had been used to hatch chicks of an infected flock. When chicks of susceptible disease-free parents were hatched in the same incubator with chicks from infected parents, a high incidence of lymphomatosis occurred in the chicks of the disease-free hen.^{14, 31}

Transmission from inoculated birds to noninoculated penmates has been repeatedly reported.^{26, 37, 38, 40-43} Factors that appear to be important in contributing to a high rate of contact transmission are (1) intimate contact of chickens of similar age (young age at exposure) and (2) a high proportion of infected chicks serving as the source of exposure.

The mechanism of contact transmission has also received some attention. It was found that VL could result from infection through any of the natural body openings. Different routes of infection with the Strain RPL 12 VL virus, as determined by experimentation, gave levels of transmission in the following descending order: tracheal, nasal, cloacal, conjunctival, oral, and aerogenic.⁴⁴ However, when the virus was deposited in the crop, VL did not occur at a significant level.

Possible natural sources of infection have also been investigated. Oral washings from naturally occurring and experimentally induced cases of VL have been found to be highly infectious.^{25, 45} Oral washings of some normal-appearing birds of an infected flock, and the oral washings of day-old chicks that were the progeny of hens of an infected flock, also were infectious, although somewhat less so than the washings from lymphomatous chickens.

Information on the relation between age and the shedding of VL virus in the saliva was obtained in an experiment in which one group of day-old chicks of a susceptible line, free from prior infection, was inoculated intraperitoneally with a strain of VL virus. A second group of the same source and age was placed in the same brooder unit. From each group, 12 chickens were randomly selected and oral washings were collected at 10, 30, 90, and 180 days of age. The individual washings for each group of chickens and for each collection were pooled, and infectivity tests were conducted. The oral washings from the inoculated chickens collected at 10 days were found to be highly infectious, and they remained so for all collection periods; whereas tests on oral washings of the contact chickens showed that the 90-day collection was the first to contain significant levels of virus, and the 180-day collection had about the same level as those of the inoculated chickens. Of the 12 inoculated birds, 7 died of VL during the age period of 98 to 236 days, and only 4 of 12 contact birds died during the 206 to 212 day age period.⁴⁵ These results definitely show that chickens may shed highly infectious levels of VL virus into their saliva soon after they have become exposed. When the exposure is definite and massive, as by inoculation, large amounts of virus appear in the saliva within 10 days. When the exposure is indefinite, as by contact infection, a longer period is required for the average level of salivary virus to attain highly infectious levels.

Feces from birds with VL and from certain normal-appearing hens also

contain infectious levels of VL virus.^{25, 45} It was also found that the infectivity of feces may be completely lost when it is mixed with feces from other birds. This cannot be accounted for on the basis of dilution factors, and it indicates the presence of a neutralizing or inactivating principle in the feces of certain birds.

It is highly significant that the saliva becomes infectious early in the life of the chicken—that is, in baby chicks when infection is derived from the egg and, within three months, when the infection is derived during the brooding period—and that the chicken remains in apparent good health for long periods thereafter. The feces are also infectious under similar conditions, but the apparent amount of virus is not as great as in the saliva, and it is less regular in its presence.

Preliminary tests have shown that hens that shed VL virus into their eggs and saliva, and day-old chicks hatched of eggs from such hens, which likewise shed virus into their saliva, also may have significant levels of serum antibodies,³⁴ suggesting little or no interference between the serum antibody and the shedding of virus into the egg or the saliva. Since such relationships have been found in many other diseases, this was not an unexpected finding.

It would now appear that the virus of VL is spread by normal-appearing hens (via the feces or droppings), by the saliva, and in the egg. Likewise, chickens with lymphoid tumors shed virus in the same manner. Baby chicks hatching from infected eggs cause a contamination of the incubator by a dissemination of dried extraembryonic fluids, feces, and other hatching egg debris. Such chicks shed virus in their saliva and feces during the brooding period and contaminate the feed, water, and other material and equipment of the brooder units, thus providing an intimate source of infection for susceptible penmates.

Since overt infection occurs after the experimental administration of virus by the oral and respiratory routes, it is likely that these routes are portals of entry of naturally disseminated virus.

Evidence that the common chick drinking fountain of the brooder unit is of major importance in the spread of the disease is shown by the results of an experiment wherein the influence of two different types of water fountains on contact transmission was studied.³⁴ It was found that the extent of transmission between chicks inoculated intraperitoneally and noninoculated chicks of the same hatch, when brooded in a pen with a rapidly flowing water fountain, was only about one third as great as the transmission in birds reared in a similar pen but with a nonflowing water fountain. Virus placed directly in a nonflowing water fountain (5 times during the first 30 hours) of a third pen produced as much VL as occurred in the contact chicks of the nonflowing water pen. These results strongly indicate that chicks infected at one day of age by intraperitoneal inoculation shed sufficient virus in their saliva to cause a significant VL virus contamination of drinking fountains when the fountain is of the reservoir type, in which contamination is allowed to accumulate. The water in such fountains may be responsible for the spread of a significant amount of VL virus to susceptible, previously non-infected chickens of the same pen.

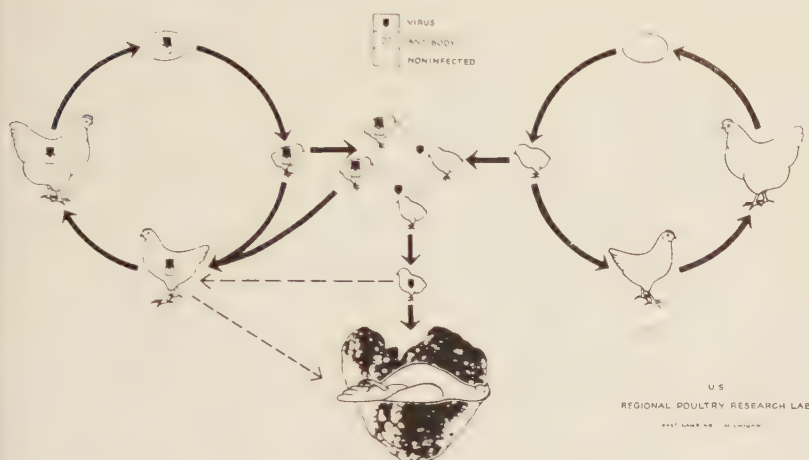


FIGURE 1. Concept for the mechanism of the natural transmission of visceral lymphomatosis.

Although this was an experimental demonstration, using inoculated chicks, it is probable that under natural conditions this mechanism of the spread of infection is of major importance, since it has been shown that day-old chicks of naturally infected hens shed virus in their saliva.

A mechanism for the natural transmission of VL is graphically presented in FIGURE 1. Chickens with an inapparent infection produce eggs from which chicks hatch that are infected yet protected by maternal antibodies. Such chicks are the source of infection for chicks of hens without inapparent infection or antibodies. Death losses from VL occur primarily in chicks without antibodies, but may also occur in the progeny of infected hens. The occurrence of losses in the latter no doubt is related to the quantitative relationships between virus and antibodies, and to other yet unknown influencing factors.

It should be assumed neither that the above is the only mechanism for the natural transmission of VL, nor that it is the mechanism of major importance for neural or ocular lymphomatosis. The studies cited have dealt almost entirely with the visceral form; little is known concerning the transmission of neural lymphomatosis, and almost nothing of the transmission of ocular lymphomatosis.

Summary

The three forms of lymphomatosis in chickens—ocular, neural, and visceral—have certain similarities in their histopathological manifestations, but appear to be caused by distinctly different viral agents. Visceral lymphomatosis must be considered a malignant neoplasm and, of the several infectious neoplasms of the chicken, it is apparently the only one that is also contagious.

Most infections with visceral lymphomatosis remain in a latent state.

Only a few chickens in a flock may die of the disease, yet a high percentage shed virus into their eggs, saliva, and feces. Such chickens also have circulating antibodies that are deposited in the egg and serve to give a certain measure of protection to their progeny. These chicks shed virus in their saliva and feces, thereby contaminating the hatching unit of the incubator and the brooder units, thus providing an excellent source of infection for other more susceptible chicks of hens without antibodies. Results of a preliminary experiment indicate that certain types of water fountains may play an important role in the mechanism of transmission in the brooder units.

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CHEMICALLY INDUCED TUMORS IN FOWL

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Cancer diagnosis and classification are based on histology, not, as in most diseases, on etiology. Consequently, however they may have originated, we accept as examples of the same disease histologically similar tumors. In mammals, spontaneous and chemically induced tumors of similar histology behave in the same way when transmitted by experimental grafting to new hosts: with very few exceptions, only intact cell grafting gives rise to tumors.

In fowls the situation is quite different. Few spontaneous carcinomata have been established as transplantable tumors, and we know almost nothing of their etiology. On the other hand, many spontaneous sarcomata and leukoses are transmissible, both by cells and by cell-free extracts and filtrates. However, most chemically induced sarcomata are not so transmissible and, like those of mammals, can be transmitted only by intact cells. A few claims of induced filterable fowl sarcomata are complicated by spontaneous leukosis in the birds used.

Burmester and Gentry (1954) have shown that carriers of visceral lymphomatosis exist in many poultry flocks. This finding must be borne in mind when assessing the significance of viruses in relation to tumors, however induced.

The classical experiments of Peyton Rous and his colleagues between 1910 and 1919 established beyond doubt that some spontaneous sarcomata of domestic fowl carry an infective agent that can reproduce the disease in a new host. Most investigators agree that these infective agents are particulate and have an average diameter of about 70 m μ , as shown by ultracentrifugation and by filtration through collodion membranes. Whether these agents should be regarded as viruses in the generally accepted sense of agents foreign to the infected species, or whether they may be subcellular, self-replicating organelles derived from previously normal constituents of the fowl's cells has not been determined.

With the advent of chemical carcinogenesis, it became possible to induce sarcoma in many species, including fowl, and such tumors have been histologically and biologically accepted as examples of malignant neoplasia. Murphy and Landsteiner (1925) first studied a transplanted tar-induced sarcoma of the fowl and showed that, since it could be propagated only by intact cells, it behaved like mammalian sarcoma and unlike the spontaneous filterable sarcoma. Many subsequent workers have confirmed this, and we are faced with two types of sarcoma in the fowl—spontaneous sarcomata, many of which are transmissible by cell-free filtrates (filterable tumors) and chemically induced sarcomata, which are not filterable. There are a few disputed exceptions, but these do not affect the general truth of this statement.

We must remember that the fowl tumors that have been most effectively

studied are those that were most successfully transmitted to new hosts, and that such success may have been dependent upon the presence in them of a causal virus. It is not surprising that this group of spontaneous tumors has yielded evidence of virus etiology.

We know nothing about the etiology of spontaneous tumors that were not transmissible even by cell grafting, and this includes almost all epithelial tumors of fowl.

The electron microscope, which can easily resolve particles of the order of 70 m μ , seemed to offer a prospect of determining whether or not the infective sarcomata contain demonstrable virus particles not present in the nonfilterable tumors. Unfortunately, however, such a simple solution of the problem has not been realized (Howatson, 1953). Claude *et al.* (1947) first demonstrated viruslike particles in the cytoplasm of Rous sarcoma No. 1, and similar particles have been illustrated in cytoplasmic vacuoles in the Rous sarcoma and in Mill Hill 2 endothelioma by several subsequent workers. All are agreed, however, that these particles are not present in the majority of tumor cells examined, and some ingenious theories have been advanced in explanation.

The contribution of my associates and myself to this vexed question is based on the study of 63 chemically induced sarcomata in several varieties of fowl over the past 26 years. Only 17 of these tumors were successfully transplanted by cell graft through more than 1 serial passage. They were not complicated by leukosis. Repeated attempts to transmit them by cell-free filtrates always failed. Moreover, less stringent procedures, that nevertheless destroy or remove most of the intact cells, have almost always failed.

Frequently we have tested our techniques by comparison between the known filter-passing tumors: Rous No. 1, Fujinami, Mill Hill 2, and the chemically induced tumors of our GRCH series 1 to 18. The results of these experiments have been published (Peacock, 1933, 1935a, 1935b, 1946; Peacock and Peacock, 1949, 1953, 1954, 1956), and they provide evidence for at least a quantitative and, I think, also for a qualitative difference between these two classes of tumor.

Recently we have also studied a transplantable tumor, GRCH 22, that arose 61 $\frac{1}{2}$ years after periodic administration of solutions of 2-acetylaminofluorene (Peacock and Peacock, 1954) by injection into the lumen of the crop. This experiment yielded a number of epithelial tumors, mostly of glandular origin, but none of them was successfully transplanted. GRCH 22 was of uncertain histogenesis, but has been classified provisionally as a lymphosarcoma. As we have not encountered a similar tumor in some hundreds of line-bred White Leghorn fowls, we regard this tumor as having been induced by 2-acetylaminofluorene. It differs biologically and histologically from all the other fowl tumors we have studied, and it grew as fast as the Rous No. 1 or the Mill Hill 2 tumors.

In one experiment the GRCH 22 tumor was transmitted successfully by homogenate filtered through No. 42 Whatman filter paper—a procedure that usually gives negative results with GRCH tumors. Subsequently we have failed with such GRCH 22 filtrates, and the early result may have been due

to the passage of a few intact cells, which are by no means certainly excluded by such simple filtration.

Our electron-microscopy studies have been limited to Rous No. 1 and Mill Hill 2 filterable tumors, and to GRCH 16 and 22 nonfilterable tumors. The results are in some ways disappointing for, although we have not seen evidence for viruslike agents in the nonfilterable tumors, as might be anticipated, we have also failed to find them in the majority of cells of undoubtedly infective tumors. Perhaps more extensive study may reveal such particles more frequently, but their absence, even in limited studies, is perplexing in the case of the infective tumors.

In contrast with the appearance of thin sections of tumors, the homogenized tumor extracts we use routinely for propagation of all types of sarcoma yield an abundance of particles of varying size, some of which may be viruses. The difficulty here is that we have no means of identifying viruses from other particles in homogenates.

The early observation of Rous *et al.* (1912), that diatomaceous earth added to tumor filtrates increased the number of successful transmissions, suggested that the well-known adsorptive capacity of diatoms might help to retain virus particles in contact with a foreign body that would induce local fibroblastic proliferation, thus providing a large susceptible cell population in intimate contact with the virus. When preparations of cell-free filtrates of Rous No. 1 sarcoma with Kieselguhr added were centrifuged, the deposit, when examined in the electron microscope, showed discrete particles adhering to the diatoms; a similar deposit was injected into test chicks that developed sarcoma.

Subsequent attempts to demonstrate such viruslike particles by adsorption on the same sample of Kieselguhr have not been consistently successful. The particles seen in this way may be partly or wholly virus, but certainty of this could be achieved only by the isolation of a single particle and proof that it could reproduce the disease. Such proof seems to be beyond our resources at present.

No such adsorbed particles were found in a recent test of GRCH 16 sarcoma.

While considering the significance of the intracytoplasmic and extracellular "virus" particles illustrated by several authors, one should surely ask what may be present in the nucleus of the tumor cell. Nuclei are too dense for direct electron microscopy of fresh preparations, and thin sections have not, as far as I know, revealed any viruslike particles, even in highly infective tumors.

Bearing in mind the frequency of secondary infection in the field of bacteriology, we should consider the possibility of saprophytic viral infection or contamination when interpreting morphologic evidence of virus infection.

Although it may be attractive to regard the viruslike particles in an infective tumor cell as the continuing cause of the tumor, the rarity of such particles does not support this concept. The theory that the virus passes through cycles of development, much of the time as unidentified subviral units, seems to me to be unsupported by evidence. Indeed, Bedson (1955)

failed recently to find any biological evidence for this concept. Beard (1956) has advanced cogent arguments against the concept of masked tumor viruses. Carr (1953) has made ingenious attempts to treat the problem mathematically, but the results of his calculations suggest that only some of the apparently identical particles are infective.

In the case of nuclear material, owing to the limitations of electron microscopy due to the density of intact nuclei and the impossibility of examining fresh living cells, we cannot correlate morphology with biological characteristics. Infective filtrates must contain both cytoplasmic and nuclear particles, however, and virus might be present in any part of the cell or in the intercellular fluids.

Wherever the virus is situated, it seems to be the continuing cause of the infective sarcoma. If this is the case, any circumstance that leads to the death or destruction of the virus should be followed by arrested growth or retrogression of the tumor. This occurs spontaneously in a small proportion of Rous sarcoma. The Fujinami sarcoma of fowl and ducks is particularly interesting in this connection. Infective filtrates of this tumor cause histologically similar tumors in fowl and ducks; however, although the fowl tumors grow progressively and in most cases kill the host, in the duck the tumor usually retrogresses spontaneously.

If the virus is the continuing cause of the tumor, one would expect to find it in the progressively growing fowl tumor, but not in the fowl or duck tumor in the retrogressing stage. Biological tests have shown this to be true. I know of no electron-microscopy studies bearing on this point, but they might be well worth pursuing.

Comparison of the free amino acid patterns of 2 filterable and 2 nonfilterable tumors with spleen, breast muscle, and 8- to 9-day chick embryos revealed more deviation from normal by the 2 chemically induced tumors, GRCH 16 and 22, than in the case of the 2 filterable tumors, Rous and Mill Hill 2. This is compatible with the idea that the virus-induced tumors differ less from normal cells than do the chemically induced tumors.

If the virus can be removed from the virus tumor cells, the cells may be able to return to normal. Perhaps this happens when spontaneous retrogression takes place. As a working hypothesis, I suggest that the chemically induced tumor cells have lost one or more of the self-replicating complexes necessary for differentiation, so that a critical increase in volume occurs and leads to cell division before differentiation has occurred.

The daughter cells, being minus the same essential complex, would go through the same process, and thus division would occur automatically and ad infinitum. Naturally one would fail to demonstrate a minus quantity in biological tests with filtrates of such tumors.

In the case of the virus-induced tumors, on the contrary, an addition has been made to the cell of a self-replicating complex that competes with normal mechanisms in such a way that, again, differentiation is inhibited, while growth in volume proceeds to a critical volume at which division follows automatically. Since the virus is also self-replicating, it may do one of five things: (1) keep step with the cell and divide with it; (2) multiply faster than

the cell can divide; (3) multiply slower than the cell and thus become lost; (4) stop multiplying; and (5) die. Retrogression would follow (4) or (5).

Progressive growth, temporary remission, or complete retrogression of virus-induced tumors could be explained in terms of the fate of the virus.

Conclusions

Ionizing radiations and some chemical carcinogens constitute natural or environmental risks common to man and animals and presumably account for some spontaneous tumors. However, many potent carcinogens are synthetic substances that do not occur outside laboratories. Tumors induced by these substances resemble spontaneous tumors histologically and are transplantable in susceptible hosts; no evidence has been found for causal viruses in most of these induced tumors.

On the other hand, viruses exist in nature, and some tumor-inducing viruses have been recovered from a number of tumors in several species of animals. Morphologic evidence for the existence of viruslike particles in some of these infective tumors has been provided by electron microscopic studies. Although compatible with this interpretation, this evidence does not constitute proof of identity of the etiologic and morphologic virus.

There may be other unsuspected etiologic factors; in my opinion, the relative importance of viruses and of known chemical and physical carcinogens in the evolution of spontaneous tumors cannot be assessed at the present time.

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Part V. Mouse Neoplasms

STUDIES ON THE NATURE AND BIOLOGICAL PROPERTIES OF A TRANSMISSIBLE AGENT CAUSING LEUKEMIA FOLLOWING INOCULATION INTO NEWBORN MICE*

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For the last several years we have been conducting experiments dealing with the cell-free transmission of mouse leukemia.¹⁻¹¹ One of the fundamental features of these studies was the use of newborn (less than 16-hour-old) suckling mice for the inoculation of the cell-free, centrifuged or filtered, leukemic extracts. When older suckling mice were used for inoculation, the results were erratic, and the development of leukemia, if it occurred at all, was considerably delayed. Normal adult mice were, with few exceptions, resistant to the inoculation of cell-free extracts.

Another fundamental feature of these studies was the striking strain specificity determining the susceptibility of the hosts to inoculation of the cell-free extracts. Some strains were resistant.⁶ Among the susceptible, certain lines, such as C57 Brown cd, were found sensitive only to the leukemogenic action of the extracts,⁵ whereas mice of other strains, such as C3H, reacted in a different manner, developing either leukemia,¹⁻³ parotid gland tumors,⁴ and or subcutaneous fibromyxosarcomas (FIGURE 1),^{6, 7, 9} and occasionally medullary adrenal tumors.^{6, 7}

Some of the leukemic extracts were very active, inducing leukemia or other types of tumors in a considerable number of injected mice after a relatively short latency; other extracts were less potent, inducing leukemia or tumors only occasionally, and after a longer latency. Among the extracts used for inoculations, some, although prepared from typical leukemias and under apparently identical experimental conditions, revealed no pathogenic potency on inoculation assays. Similar results have been obtained recently by Woolley.¹²

Results of Inoculation of Filtered Ak Leukemic Extracts into Newborn C3H or C3H(f) Mice

The leukemic extracts were prepared from Ak female mice that developed leukemia spontaneously or, in a few instances, from young adult Ak males and females that developed leukemia as a result of implantation of Ak leukemic cells. Under ether anesthesia, livers (care being taken to exclude the gall bladder), spleens, mesenteric tumors, and peripheral lymph nodes were removed aseptically, cut with small scissors, and ground by hand in a porcelain mortar, with sterile (chilled to 0° C.) physiological sodium chloride

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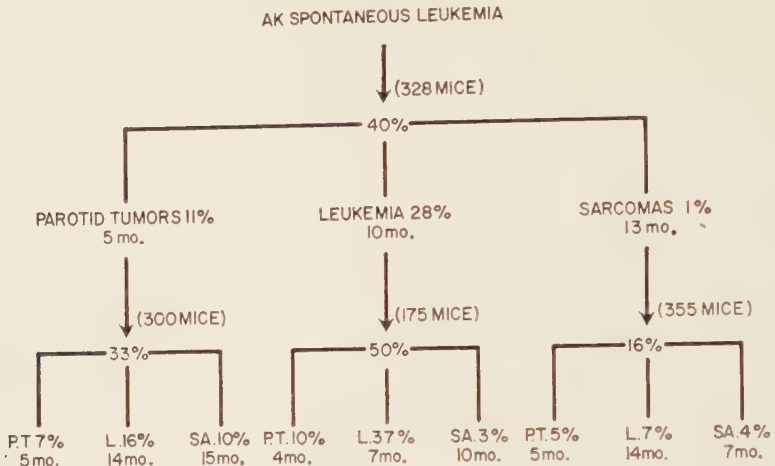


FIGURE 1. Results of the inoculation of cell-free (centrifuged or filtered) extracts into newborn C3H mice. The filtered (Berkefeld or Selas) leukemic extracts were first prepared from Ak leukemic mice and inoculated into 328 newborn C3H or C3H(f) mice. Of these, a total of 40 per cent developed either leukemia or tumors, as follows: (1) 28 per cent, leukemia; (2) 11 per cent, parotid tumors; and (3) 1 per cent, sarcomas. From the leukemias (1) thus induced, cell-free (centrifuged or filtered) extracts were again prepared and inoculated into 175 newborn C3H or C3H(f) mice. As a result, a total of 50 per cent developed either leukemia or tumors, as follows: 37 per cent, leukemia; 10 per cent, parotid tumors; and 3 per cent, sarcomas. When cell-free (centrifuged or filtered) extracts were prepared from the induced parotid tumors (2) and inoculated into 300 newborn C3H or C3H(f) mice, a total of 33 per cent developed either leukemia or tumors, as follows: 16 per cent, leukemia; 7 per cent, parotid tumors; and 10 per cent, sarcomas. Finally, when cell-free (centrifuged or filtered) extracts were prepared from the induced sarcomas (3) and inoculated into 355 newborn C3H or C3H(f) mice, a total of 16 per cent developed either leukemia or tumors, as follows: 7 per cent, leukemia; 5 per cent, parotid tumors; and 4 per cent, sarcomas.

solution added to obtain cell suspensions of 20 per cent concentration. These extracts were centrifuged in a Refrigerated International Centrifuge PR-1 at 3000 rpm ($1400 \times g$) for 15 min. at 0°C .; the supernate was then removed and centrifuged at 9500 rpm ($7000 \times g$) at 0°C . for 5 to 10 min. The final supernate was passed through Berkefeld N, or Selas (porosity 02 or 03) microporous filter candles under a vacuum pressure of 20 to 25 mm. of mercury. Each filter was tested, before and after filtration, and found to be impervious to *Escherichia coli*. In most experiments the centrifuged leukemic extracts (10 to 15 cc.) were mixed, prior to filtration, with 0.5 to 1 cc. of a 1:2000 dilution of a fresh broth culture of *E. coli*, and then were passed through the filter candles. The filters retained the *E. coli*, since the filtrates were found to be sterile, as evidenced by the inoculation of tryptose phosphate broth media (the inoculated media were incubated at 37°C . for 48 hours and then inspected for *E. coli* growth). The filtered extracts were immediately placed in sterile glass tubes immersed in larger glass containers filled with ice cubes and kept in refrigerators. The extracts were thus kept at 0°C . for periods not exceeding 48 hours prior to inoculation.

Newborn C3H or C3H(f) mice, both of the Bittner substrain, free from the mammary carcinoma agent (by foster nursing) were used. Only mice less than 16 hours old were used for inoculation; among these, many were less than 6 hours old, and most were less than 12 hours old (average age at inoculation, 10 hours).

A total of 70 filtered extracts was prepared, each from a different Ak leukemic donor. Of these extracts, 18 were found inactive on inoculation. Of a total of 328 mice inoculated with the filtered leukemic extracts, 92 (28 per cent) developed leukemia at an average age of 10 months; 36 (11 per cent) developed parotid tumors at an average age of 5 months; 3 (1 per cent) developed subcutaneous sarcomas at an average age of 13 months. A total of 40 per cent of the injected mice developed either leukemia, parotid tumors, or sarcomas (TABLE 1). The incidence of leukemia appeared higher in the foster-nursed C3H(f) mice, as compared with those carrying the milk agent. Furthermore, the incidence was higher in females as compared with the males. The data referring to these details are not sufficiently extensive, however, to warrant any definite conclusions as to the possible influence of the mammary tumor agent and the sex of the host on the incidence of the induced leukemia.

When 166 litter-mate controls were injected simultaneously with heated (65° to 68° C.) leukemic filtrate, 2 developed leukemia at 17 months of age; however, 5 mice developed parotid tumors. It is possible that heating to 65° to 68° did not completely inactivate the extracts. The possibility of contact infection should also be considered since, after injection, these mice were placed in the same nests with their litter mates that had been injected simultaneously with fresh extracts. The average age of the control mice dying free from leukemia or tumors was 16 months.

In the untreated mice of our colonies of C3H or C3H(f) mice the incidence of spontaneous leukemia is very low, not exceeding 0.5 per cent, and parotid tumors or subcutaneous sarcomas, for all practical purposes, are nonexistent.⁶

Leukemia induced in C3H mice with the filtered Ak agent was readily transplantable by cell graft into mice of the same C3H inbred line, causing acute leukemia within 2 to 3 weeks in practically all the inoculated animals. Only rarely would such transplantation succeed when the induced C3H leukemia was grafted to adult mice of the donor Ak strain.³⁻¹¹ The specificity of the transplantation experiments was striking, because leukemia induced with the cell-free Ak agent in C3H mice of the Bittner substrain was readily transplantable by graft to adult mice of the same substrain. The results were negative, however, when such leukemia was transplanted to C3H mice of the National Cancer Institute subline.

The parotid tumors induced in C3H mice by inoculation of the cell-free Ak leukemic extracts could be transplanted, using tumor cell-suspension, under the skin of C3H or C3H(f) mice. The results were only occasionally positive; tumors developed at the site of implantation in from 2 weeks to 3 or 4 months. Attempts to transplant the same tumors under the skin of adult Ak mice were negative, but succeeded in a few instances when newborn (less than 12 hours old) AK mice were inoculated.^{4, 7, 8}

TABLE 1
RESULTS OF INOCULATION OF FILTERED* AK LEUKEMIC EXTRACTS INTO NEWBORN C3H OR C3H(f) MICE†

Fresh filtrate																			Heated (65° to 68° C. ½ hr.) filtrate																		
Strain	Sex	No. mice inoc.	No. dev. leuk.	Avg. inc. (%)	Avg. age (mo.)	No. dev. paratid tumors	Pa- tid inc. (%)	Avg. age (mo.)	No. dev. sub- sar- comas	Total leuk. and tu- mor inc. %	No. of mice inoc.	No. dev. leuk.	Leuk. inc. (%)	Avg. age (mo.)	No. dev. paratid tumors	Pa- tid inc. (%)	Avg. age (mo.)	No. dev. sub- sar- comas	No. died free from leuk. or tumors (mo.)	Avg. age died free from leuk. or tumors (mo.)																	
C3H.....	F	90	22	24	9	13	14	4	0	—	39	0	0	—	4	0	—	0	39	—																	
C3H.....	M	53	10	19	12	15	28	5	2	4	51	2	4	12	5	2	4	2	51	—																	
Total C3H.....		143	32	22	10	28	20	6	2	1	43	2	1	12	43	2	1	12	43	—																	
C3H(f).....	F	111	39	35	11	6	5	3	1	1	41	1	1	16	4	1	1	1	41	16																	
C3H(f).....	M	74	21	28	9	2	3	5	0	0	31	0	0	—	—	—	—	—	31	—																	
Total C3H f		185	60	32	10	8	4	4	1	—	57	2	2	16	37	4	4	4	0	15																	
Total.....	F	201	61	30	10	19	10	4	1	—	40	—	—	—	—	—	—	—	—	—																	
Total.....	M	127	31	24	10	17	13	7	2	—	39	—	—	—	—	—	—	—	—	—																	
Grand total.....		328	92	28	10	36½	11	5	3	1	40	166	2	1	17	5	3	5	0	159																	

Some of the data in this table have been published previously.³ However, these experiments have been continued, with few animals added; additional mice developed leukemia, paratid tumors, or sarcomas. The adjuvanted data reflect the results available at this time. The mice that died when less than 2 months old were not included in the tabulation. This concerns particularly the mortality; usually, however, not exceeding 20 to 25 per cent, occurring among the infant mice within hours or days after inoculation.

*Seventy extracts were prepared, each from a different donor: 44 from Ak donors with spontaneous leukemia and 26 from Ak donors with transplanted Ak leukemia. The extracts (20 per cent cone.) prepared from leukemic organs, were centrifuged (7000 × g), and then filtered. Either Berkeley N or Selas (porosity 02 or 03) filter candles were used. Of the total of 70 filtrates prepared, 40 Berkeley, and 40 Selas, only 52 proved to be active on inoculation.

†All mice used for inoculation in this series were of the Birnair substrain at the C3H line. These designated by the symbol C3H f were of the same substrain, however, free from the mammary tumor agent by inbreeding. Most mice were less than 12 hours old, many less than 6 hours old, and none more than 16 hours old, at the time of inoculation. The average age at inoculation was 10 hours. All inoculations were subcutaneous (0.1 cc. each). The extracts were used for inoculation within 48 hours having been kept in the meantime in sterile tubes immersed in ice water, at 0° C. The heated (65° to 68° C., ½ hour) extracts were inoculated simultaneously into litter-mate control mice.

‡Among the mice that developed paratid gland tumors, several also developed subcutaneous fibrosarcomas, usually near the groin, axilla, or within the abdominal wall. In one mouse a medullary adrenal tumor was also observed. Two mice developed, in addition to paratid gland tumors, carcinomas of the submaxillary gland, arising in small multiple foci. In one instance bilateral paratid gland carcinomas developed simultaneously with leukemia.

Attempt to Recover Leukemic Agent from Organs of C3H Mice in Which Leukemia had been Induced with Filtered Ak Leukemic Extracts

It was of great interest to determine whether the leukemic agent could be recovered in its cell-free, pathogenic form from induced C3H leukemias. Accordingly, C3H mice that developed leukemia as a result of inoculation of filtered Ak leukemic extracts were sacrificed, and from their organs (livers, spleens, mediastinal and mesenteric tumors) cell suspensions of 20 per cent concentration were prepared and centrifuged in the usual manner, that is, 3000 rpm ($1400 \times g$) for 15 min., and then 9500 rpm ($7000 \times g$) for 5 to 10 min. Some of the extracts were then filtered through Berkefeld N or Selas (porosity 02 or 03) filter candles. The centrifuged ($7000 \times g$) or filtered extracts were inoculated into newborn C3H or C3H(f) mice of the Bittner substrain (average age at inoculation, 10 hours). Twenty-five extracts were prepared, each from a different C3H leukemic donor. Six

TABLE 2
RESULTS OF INOCULATION OF CENTRIFUGED OR FILTERED EXTRACTS* PREPARED FROM INDUCED C3H OR C3H(f) LEUKEMIA INTO NEWBORN C3H OR C3H(f) MICE†

Strain	Sex	No. of mice inoc.	No. dev. leuk.	Leuk. inc. (%)	Avg. age leuk. dev. (mo.)	No. dev. parotid tumors	Parotid tumors inc. (%)	Avg. age parotid tumors dev. (mo.)	No. dev. subcu. sarcomas	Sarcomas inc. (%)	Avg. age sarcomas dev. (mo.)	Total leuk. and tumors inc. (%)
C3H.....	F	54	20	37	6	5	9	4	1	2	11	48
C3H.....	M	49	13	27	10	3	6	3	4	8	10	41
Total C3H.....		103	33	32	8	8	8	3	5	5	11	45
C3H(f).....	F	35	13	37	8	6	17	4	0	—	—	54
C3H(f).....	M	37	17	46	7	3	8	4	0	—	—	54
Total C3H(f) ..		72	30	42	8	9	13	4	0	—	—	54
Total.....	F	89	33	37	7	11	12	4	1	1	11	50
Total.....	M	86	30	35	8	6	7	4	4	5	10	47
Grand total....		175	63	36	7	17‡	10	4	5	3	10	49§

Some of the data in this table have been published previously.¹⁰ These experiments have been continued, however, with few animals added; additional mice developed leukemia, parotid tumors, or sarcomas. The adjusted data reflect the results available at this time. The mice that died when less than 2 months old were not included in the tabulation. This concerns particularly the mortality, usually, however, not exceeding 20 to 25 per cent, occurring among the infant mice within hours or days after inoculation.)

* The extracts, prepared from leukemic organs of C3H donor mice (in which leukemia was induced by inoculation of cell-free Ak leukemic extracts, were centrifuged ($7000 \times g$) or filtered (Berkefeld N or Selas 03 or 02). Of 25 extracts, each from a different donor, 6 were inactive on inoculation.

† All C3H mice used for inoculation, were of the Bittner substrain. Those designated by symbol C3H(f) were of the same substrain, however, free from mammary tumor agent by foster nursing. Most mice were less than 12 hours old, many less than 6 hours old, and none more than 46 hours old at the time of inoculation. The average age at inoculation was 10 hours. All inoculations were subcutaneous (0.1 cc. each). The extracts were used for inoculation within 48 hours, having been kept at 0° C.

‡ Two mice developed parotid tumors and leukemia. Four mice developed parotid tumors and subcutaneous fibromyxosarcomas.

§ At an average age of 13 months, sixty mice died free from leukemia or tumors, and thirty mice were still alive and free from leukemia or tumors.

extracts were found inactive on inoculation (TABLE 2). Of a total of 175 mice inoculated, 63 (36 per cent) developed leukemia at an average age of 7 months; 17 (10 per cent) developed parotid tumors at an average age of 4 months; and 5 (3 per cent) developed subcutaneous sarcomas at an average age of 10 months. A total of 49 per cent developed either leukemia, parotid tumors, or sarcomas (TABLE 2, FIGURE 1). Apparently therefore, extracts prepared from C3H mice with induced leukemia were more potent than those prepared from Ak mice with spontaneous or transplanted leukemia; the incidence of leukemia resulting from injection of the C3H leukemic extracts was higher and the latency was shorter, as compared with the potency of the Ak leukemic filtrates.

Attempt to Recover Oncogenic Agents from Induced Parotid Tumors

An attempt was made to recover the agent, or agents, from induced parotid tumors. C3H or C3H(f) mice with induced parotid tumors were used as donors. The parotid tumors developed in these mice as a result of inoculation within 16 hours after birth of cell-free Ak or C58 leukemic extracts, or cell-free extracts prepared from leukemia-induced parotid tumors. The parotid tumors were dissected aseptically, weighed, and then ground by hand in a mortar with chilled sterile saline solution added to make a cell suspension of 20 per cent concentration. These cell suspensions were centrifuged at 3000 rpm ($1400 \times g$) for 15 min. and then at 9500 rpm ($7000 \times g$) for 5 to 10 min. The final supernate was used for inoculation, or was passed through Selas 02 or 03 filter candles and then injected. New-born C3H mice of the Bittner substrain were used for inoculation (average age at inoculation, 9 hours). Thirty-eight extracts were prepared, each from a different parotid tumor. Of these, 10 were found inactive on inoculation (TABLE 3). Of 300 injected mice, 47 (15.6 per cent) developed leukemia at an average age of 14 months; 20 (6.7 per cent) developed parotid tumors at an average age of 5 months; and 29 mice (10 per cent) developed subcutaneous sarcomas at an average age of 15 months. The total incidence of leukemia and tumors was 32 per cent.

Attempt to Recover Oncogenic Agents from Induced Sarcomas

Subcutaneous fibrosarcomas (FIGURE 2), fibromyxosarcomas, giant-cell tumors, and the like, were induced in C3H or C3H(f) mice with centrifuged or filtered, either leukemic or parotid tumor extracts. An attempt was then made to recover oncogenic agents from these sarcomas. It was rather difficult to prepare extracts from these hard, fibrous tumors. After the tumors were dissected aseptically by sharp scissors, they were weighed, cut into small pieces, and then ground, first by hand in a mortar, and then in a VirTis "45" high-speed homogenizer for 3 min. The resulting, usually viscous, cell-suspension was first centrifuged at 3000 rpm ($1400 \times g$) at 0°C . for 15 min. The supernate was then centrifuged at 9500 rpm ($7000 \times g$) for 5 to 10 min. The final supernate was used for inoculation (10 extracts), or was passed first through Selas (porosity 02 or 03) filter candles and then injected (25 filtrates). Newborn C3H mice of the Bittner substrain were

TABLE 3
RESULTS OF INOCULATION OF CENTRIFUGED OR FILTERED EXTRACTS PREPARED FROM LEUKEMIA INDUCED PAROTID TUMORS* INTO NEWBORN C3H OR C3H(f) MICE†

Strain	Sex	No. of mice inoc.	No. leuk. dev.	Leuk. inc. (%)	Avg. age leuk. dev. (mo.)	No. dev. parotid tumors	Parotid tumors inc. (%)	Avg. age parotid tumors dev. (mo.)	No. dev. sarcomas	Sarcomas inc. (%)	Avg. age sarcomas dev. (mo.)	Total leuk. and tumors inc. %	No. died free from leuk. or tumors	Avg. age died free from leuk. or tumors (mo.)
C3H.....	F	130	28	22	13	4	3	8	9	7	15	32	89	17
C3H.....	M	103	9	9	14	2	2	7	11	11	14	22	81	14
Total C3H.....		233	37	16	14	6	2.5	8	20	8.5	14	27	170	15.5
C3H(f).....	F	32	8	25	13	3	9	4	7	22	16	56	14	19
C3H(f).....	M	35	2	6	16	11	31	4	2	6	12	43	20	15
Total C3H(f).....		67	10	15	13	14	21	4	9	13	15	49	34	16.5
Total.....		162	36	22	13	7	4	9	16	10	15	36	103	17
Total.....		138	11	8	14.5	13	9	4	13	9	14	26	101	14
Grand Total.....		300	47	15.6	14	20‡	6.7	5	29	10	15	32	204	16

Some of the data in this table have been published previously.⁷ However, these experiments have been continued, with few animals added; additional mice developed leukemia, parotid tumors, or sarcomas. The adjusted data reflect results available at this time. Those mice that died when less than 2 months old were not included in the tabulation. This concerns particularly the mortality, usually, however, not exceeding 20 to 25 per cent, occurring among the infant mice within hours or days after inoculation.

* The parotid tumors were induced in C3H or C3H(f) mice, and resulted from inoculation, within 16 hours after birth, of cell-free Ak or C58 leukemic extracts, or cell-free extracts prepared from leukemia-induced parotid tumors. The extracts prepared from parotid tumors were either centrifuged (7000 \times g) or filtered (Selas 02 or 03). Of a total of 38 extracts, each prepared from a different donor, 10 were found to be inactive on inoculation.

† All mice used for inoculation in this series were of the Bittner substrain of the C3H line (average age at inoculation, 9 hours). A total of 74 litters was injected.

‡ Of the 20 mice that developed parotid tumors, 6 developed subcutaneous sarcomas also.



FIGURE 2. Subcutaneous fibrosarcoma. C3H(f) male No. 512, Exp. 2488^A, was inoculated when it was less than 14 hours old with a centrifuged ($7000 \times g$) extract prepared from normal Ak embryos. As a result, at the age of 7 months this mouse developed bilateral parotid gland carcinomas and, 2 months later, in the vicinity of the left inguinal pit, a subcutaneous fibrosarcoma. Hard, and difficult to cut, this tumor infiltrated the surrounding tissues. Hematoxylin-eosin stain. $\times 300$.

used for inoculation (average age at inoculation, 9 hours). Although these experiments are still in progress, the results obtained thus far suggest that cell-free extracts obtained from subcutaneous sarcomas and injected into newborn C3H mice are capable of inducing either leukemia, parotid tumors, or subcutaneous sarcomas (TABLE 4). A total of 35 extracts was prepared, each from a different donor. Of these, 18 extracts were found to be inactive

TABLE 4
RESULTS* OF INOCULATION OF CENTRIFUGED OR FILTERED EXTRACTS PREPARED FROM INDUCED SARCOMAS† INTO NEWBORN C3H OR C3H(f) MICE

No. of extracts‡	Avg. age at inoc. (hr.)	No. of mice inoc.	No. Leuk. inc. (%)	Avg. age leuk. dev. (mo.)	No. dev. parotid tumors‡	Parotid tumors inc. (%)	Avg. age parotid tumors dev. (mo.)	No. dev. subcu. sar- comas	Sar- comas inc. (%)	Avg. age sar- comas dev. (mo.)	Total leuk. and tumors inc. (%)	No. died free from leuk. or tumors	Avg. age died free from leuk. or tumors (mo.)	No. alive and free from leuk. or tumors	Avg. age alive free from leuk. or tumors (mo.)
35	9	355	26	7	14	19‡	5	5	4	7	16	141	16	157	11

Those mice that died when less than 2 months old were not included in the tabulation. This concerns particularly the mortality, usually, however, not exceeding 20 to 25 per cent, occurring among the infant mice within hours or days after inoculation.

* These experiments are still in progress at this time.

† The subcutaneous sarcomas were induced in C3H or C3H(f) mice with cell-free (either leukemic or parotid tumor) extracts. From these sarcomas, either centrifuged (7000 \times g) or filtered (Selas 02 or 03) extracts were prepared. Of 35 extracts, each from a different tumor, 18 were found to be inactive on inoculation.

‡ One mouse developed bilateral parotid tumors and also a medullary adrenal tumor.

on inoculation (TABLE 4). A total of 355 mice was inoculated; as a result, 20 (7 per cent) developed leukemia at an average age of 14 months; 19 mice developed parotid tumors (5 per cent) at an average age of 5 months; 13 mice (4 per cent) developed subcutaneous sarcomas at an average age of 7 months. The total incidence of either leukemia or tumors was 16 per cent (FIGURE 1).

Attempt to Transplant the Induced Sarcomas

Thirty-four subcutaneous sarcomas induced in the C3H or C3Hf mice with the leukemic extracts were transplanted into C3H mice. Twenty-eight tumors were used for the preparation of cell suspensions. These suspensions were ground by hand, or in the VirTis "45" homogenizer (3 min.), and then were injected subcutaneously (0.5 to 1 cc. each) under the skin of 53 C3H mice. Of the 28 sarcomas, only 10 could be transplanted successfully; 19 of the 53 infected C3H mice developed tumors at the site of implantation after a latency varying from 2 weeks to 5 months (average 2 months). When, however, in 6 additional experiments the sarcomas were transplanted by grafting tumor pieces, transplantation succeeded in all 6 instances (average latency 2 months). In these 6 experiments, pieces of tumors approximately 5 mm. in diameter were introduced aseptically under the skin, through an incision made in the skin on the back of 11 C3H mice. The incisions were then closed with sutures. All 11 mice developed tumors at the site of implantation, 10 after a latency varying from 1 to 2 months, and 1 after 4½ months. All tumors developing at the site of the injection of the tumor cell suspensions, or at the site of implantation of fragments of tumors, were hard, fibrous, solid sarcomas of a morphology essentially similar to that of the donor tumors, which were fibrosarcomas, fibromyxosarcomas, giant-cell tumors, and the like. Attempts to transplant the C3H sarcomas into adult Ak mice were not successful.

Experiments on the Nature of Parotid Tumors Which Had Been Induced with Normal Organs or with Either Heated or Ether-Treated Leukemic Extracts

In previous experiments it was observed that parotid tumors could be induced also with C3H or C57 Brown normal organ extracts.⁷ When cell-free extracts, prepared from healthy C3H or C57 Brown cd embryos, or from pooled organs (liver, spleen, heart, kidneys, lungs) prepared from normal healthy adult C3H or C3Hf mice or from normal guinea pigs, were injected into newborn C3H or C3Hf mice, parotid tumors could be induced in a few instances (FIGURE 3).⁷

It was also observed that heated (58° to 68° C., one-half hour),^{6, 13} centrifuged or filtered leukemic extracts injected into newborn C3H mice occasionally induced parotid tumors. Furthermore, leukemic extracts treated with ether *in vitro*¹⁴ lost their potency to induce leukemia, but could still induce either parotid tumors or sarcomas.

It was necessary to consider the possibility that a parotid tumor agent, perhaps distinct from the leukemic virus, was present in some of the normal, healthy mice of low-leukemic lines, such as C3H or C57 Brown cd and, furthermore, that such an agent could be more resistant than the leukemic

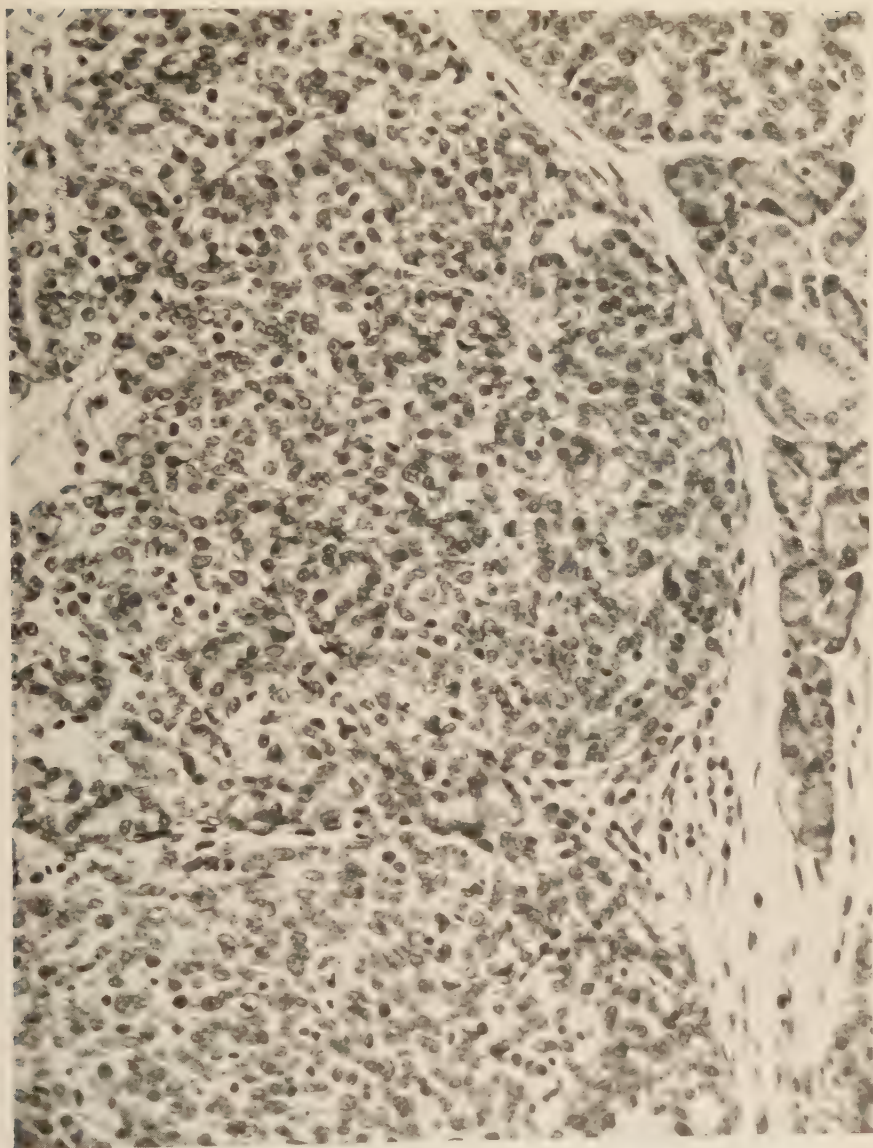


FIGURE 3. Parotid carcinoma. A centrifuged ($7000 \times g$) extract was prepared from the normal organs (kidneys, spleens, livers) of young healthy C3H mice, and the supernate was lyophilized. The dry-frozen extract was then preserved in a sealed ampoule at $+4^{\circ}\text{C}$. in a refrigerator for 6 weeks. After this lapse of time the extract was resuspended in sterile physiological saline and injected into 2 newborn C3H mice less than 15 hours old. Only one of these 2 mice survived, C3H male No. 111, Exp. 3218¹. At the age of $3\frac{1}{2}$ months this mouse developed typical bilateral parotid gland carcinomas. Hematoxylin-eosin stain. $\times 300$.

TABLE 5

RESULTS OF INOCULATION INTO NEWBORN C3H OR C3H(f) MICE* OF CENTRIFUGED (7000 × g) EXTRACTS PREPARED FROM PAROTID TUMORS INDUCED WITH NORMAL ORGANS, OR WITH HEATED OR ETHER-TREATED LEUKEMIC EXTRACTS)

No. of tract prep.	Donor's parotid tumors resulted from inoc. of	Strain inoc.	Sex	No. of mice inoc.	No. dev. leuk.	No. dev. parotid tumors	Avg. age parotid tumors dev. (mo.)	No. dev. subcut. sarcomas	Avg. age sarcomas dev. (mo.)	No. died free leuk. or tumors	Avg. age died free leuk. or tumors	No. alive free from leuk. or tumors	Avg. age alive free from leuk. or tumors
9	Normal C3H or C57 Brown organs ctr.	C3H	F	7	0	0	0	2	16				
		C3H	M	21	0	1	10	5	12				
		C3H(f)	F	14	0	1	3	0					
2	Heated 58°C., ½ hr. leuk. ctr.	C3H(f)	M	16	0	0	0	0					
		C3H(f)	F	9	0	0	0	0					
		C3H(f)	M	3	0	0	0	0					
1	Ether-treated leuk. ctr.	C3H(f)	F	2	0	0	0	0					
		C3H(f)	M	2	0	0	0	0					
12	Total			74	0	2	6	7	13	40	16	25	11

Those mice that died when less than 2 months old were not included in the tabulation. This concerns particularly the mortality, usually, however, not exceeding 20 to 25%, occurring among the infant mice within hours or days after inoculation.

* All mice used for inoculation in this series were of the Birtnur sub-strain of the C3H line. Those designated by the symbol C3H(f) were of the same sub-strain, however free from the mammary tumor agent by foster nursing. Most mice were less than 12 hours old, many less than 6 hours old, and none more than 16 hours old at the time of inoculation. The average age at inoculation was 8 hours.

virus to heating or to ether treatment. Should such an assumption be correct, it would then follow that extracts prepared from normal parotid tumors induced with normal organs, or tumors that had been induced with heated or ether-treated leukemic extracts, would contain, if any, only a small amount of the leukemic agent, but would still be capable of inducing parotid tumors. Accordingly, 12 cell-free extracts were prepared from such parotid tumors and were injected into 74 newborn C3H mice (TABLE 5). None developed leukemia, but 2 developed parotid tumors, and 7 developed subcutaneous sarcomas. Although the number of animals injected in this series was relatively small, and no definite conclusions can yet be drawn, these results are consistent with the assumption that the parotid tumor agent is distinct from the agent causing leukemia. It is interesting to compare these results with those summarized in TABLE 3. When extracts were prepared from leukemia-induced parotid tumors, and were injected into newborn C3H mice, 15.6 per cent of the inoculated mice developed leukemia.

It is possible, therefore, that certain parotid tumors may also contain, either as a passenger virus or as an oncogenic companion with a pathogenic potential, a leukemic agent actually distinct from the parotid tumor agent. Such a leukemic agent, although present in the tumor, may not reveal itself by any pathogenic action in the carrier host, although it may become active when transferred to another host. An extract prepared from such a parotid tumor may then induce leukemia.

Other parotid tumors, however, while microscopically indistinguishable, may be relatively or completely free from the leukemic virus. Extracts prepared from such parotid tumors may not induce leukemia, but may still be capable of inducing either parotid tumors or sarcomas (TABLE 5).

Ultracentrifugation of Leukemic Extracts

In an attempt to sediment the oncogenic agents, the following series of experiments was carried out: 9 Ak females with spontaneous leukemia and 15 C3H females with Ak-agent-induced leukemia were used as donors for the preparation of leukemic extracts. The extracts of 20 per cent concentration were prepared in the usual manner, from livers, spleens, mediastinal and mesenteric tumors, and peripheral nodes. After initial centrifugation of 3000 rpm ($1400 \times g$) for 15 min., the supernate was centrifuged at 9500 rpm ($7000 \times g$) for 5 to 10 min. The $7000 \times g$ supernate was then used for final centrifugation in a Spinco Model L Ultracentrifuge at 40,000 rpm, using a Swinging Bucket Rotor SW 39 (maximum $173,000 \times g$, average $125,000 \times g$). The ultracentrifugation at 40,000 rpm was for periods of time varying from 15 to 150 min. (this time refers to the actual centrifugation at top speed, that is, at 40,000 rpm; add 3 to 4 min. each, for acceleration and for deceleration). Only the middle part of the final supernate was then carefully removed from the tubes with a syringe; the rest of the supernate was discarded. The sediment pellet was resuspended in a small quantity (1.5 to 2.5 cc.) of sterile saline. The supernate and the resuspended sediment, kept at 0°C. were used for inoculation within 48 hours after preparation. New-

TABLE 6
RESULTS OF INOCULATION INTO NEWBORN C3H OR C3H₁₀₁ MICE OF CELL-FREE LEUKEMIC EXTRACTS AFTER ULTRACENTRIFUGATION AT 40,000 RPM* FOR PERIODS OF TIME VARYING FROM 15 TO 150 MIN.

Centrif. 40,000 rpm min. [†]	Sediment						Supernate						
	No. of extracts	No. of mice inoc.	No. dev. leuk.	Avg. age (mo.)	No. dev. parotid tumors	Avg. age (mo.)	No. of mice inoc.	No. dev. leuk.	Avg. age (mo.)	No. dev. parotid tumors	Avg. age (mo.)	No. dev. sarcomas	Avg. age
15	7	40	21	5	4	7	2	9	5	0	0	0	0
30	3	26	3	4	0	0	0	0	0	0	0	0	0
35	1	1	1	6	0	0	0	0	0	0	0	0	0
45	3	5	0	0	0	0	0	0	0	0	0	0	0
60	5	13	4	8	0	0	2	13	0	0	0	0	0
90	1	4	0	0	1	8	1	15	0	0	0	0	0
120	2	8	5	12	0	0	0	0	14	0	0	0	0
150	2	3	0	0	0	0	1	16	0	0	0	1	16

The mice that died when less than 2 months of age were not included in the tabulation. This concerns particularly the mortality, usually, however, not exceeding 20 to 25 per cent, occurring among the infant mice within hours or days after inoculation.

* Nine Ak females with spontaneous leukemia and 15 C3H females with Ak agent induced leukemia were used as donors for the preparation of leukemia extracts. The initial centrifugation was 3000 rpm (1400 \times g) for 15 min., and then 9500 rpm (7000 \times g) for from 5 to 10 min. The 7000 \times g supernate was used for the final centrifugation in the Spinco Model L Ultracentrifuge at 40,000 rpm, using a Swinging Bucket Rotor SW 39 (average speed, 125,000 \times g).

† The time in minutes refers to the actual centrifugation at top speed (40,000 rpm). Add 3 to 4 min. each, for acceleration and for deceleration.

born C3H or C3H(f) mice of the Bittner subline were used for inoculation (average age at inoculation, 9 hours).

It is evident from the results summarized in TABLE 6 that, after centrifugation at 40,000 rpm for 30 min. or longer, only the sediment pellet was pathogenic. The supernate was, with rare exception, inactive on inoculation assays. The pathogenic factors, present in the extracts, could accordingly be sedimented in the ultracentrifuge in a relatively short time.

Apparently, therefore, the active factors were particulate components. Under ideal conditions, using the Swinging Bucket Rotor SW 39 in the Spinco Model L Ultracentrifuge at maximum speed (40,000 rpm), the sedimentation time for average biological particles of 100 m μ in diameter was 7 min. After centrifugation for 15 min. at maximum speed, biological particles of 70 m μ in diameter should be sedimented. After centrifugation for 30 min. at maximum speed, using the same SW 39 motor, biological particles of approximately 50 m μ should be sedimented. Since, after 15 min. centrifugation, the supernate was still active, but had no activity after centrifugation of 30 min. (TABLE 6), it appears that the size of the leukemogenic particles should be placed somewhere between 50 and 70 m μ . Further observations, however, are necessary, since these experiments are still in progress.

Serial, Cell-Free Transmission of the Leukemic Agent from Host to Host

Thus far, by using cell-free extracts, the leukemic agent could be transmitted from one host to another serially for at least eight successive passages without loss of potency. This is evident from TABLE 7, which summarizes the passages thus far obtained. It is also apparent that the leukemic agent, after serial passage, is now able to induce leukemia after a relatively short

TABLE 7
PASSAGE OF LEUKEMIC AGENT* THROUGH SUCCESSIVE CELL-FREE
INOCULATIONS OF NEWBORN HOSTS

Passage No.*	Extract used for inoc.	Strain inoc.	Sex	Age at inoc. (<hr.)	Route of inoc.†	Age leuk. result (mo.)
1	Fil.	C57Br	M	12	S.C.	7
2	Fil.	C3H	F	15	S.C.	4
3	Fil.	C3H(f)	F	10	S.C.	3½
4	Ctr.	C3H	F	3	S.C.	3
5	Ctr.	C3H(f)	F	4	S.C.	4
6	Ctr.	C3H	M	1	S.C.	3
7	Ctr.	C3H(f)	M	15	I.P.	1
8	Fil.	C3H(f)	F	6	I.C.	<4

* The first filtered extract prepared from a 14 months old C58 female with spontaneous leukemia.

† Symbols: S.C., subcutaneous; I.P., intraperitoneal; I.C., intracranial.

latency. At the present time the "passage virus" injected into newborn C3H mice induces leukemia in 44 per cent of the injected C3H mice.*

Preservation of the Leukemic Agent at -70° C.

In previous experiments it was reported that the leukemic agent could be preserved by lyophilization, and in 50 per cent glycerin. Thus, when leukemic cell suspensions were either lyophilized or preserved in 50 per cent glycerin for periods of up to 15 months and then resuspended and, after centrifugation, inoculated into newborn C3H or C3H(f) mice, leukemia, parotid tumors, or sarcomas resulted.

In a series of experiments still in progress,¹³ an attempt has been made to determine whether the leukemic extracts could be preserved in dry ice (CO_2) at -70° C. Accordingly, leukemic cell suspensions were prepared in the usual manner from either leukemic Ak donors or C3H donors with Ak-agent-induced leukemia. The suspensions were placed in sterile tubes which were either sealed or tightly plugged and then quickly frozen in dry ice at -70° C. After periods varying from 2 weeks to 2 months, the tubes were removed from dry ice and defrosted within a few minutes in tap water. The defrosted cell suspensions were centrifuged at 3000 rpm for 15 min., and then at 95,000 rpm for 5 min. The final supernate was inoculated into newborn (less than 16-hour-old) C3H or C3H(f) mice. The results obtained thus far suggest that, at -70° C., the extracts remained active for at least 54 days without any apparent loss of their pathogenic potency. Such extracts, when defrosted and inoculated into newborn C3H or C3H(f) mice, were capable of inducing either leukemia, parotid tumors, or subcutaneous sarcomas. In more recent experiments it has been found that the leukemic agent can be preserved at -70° C. in sealed ampouls containing leukemic filtrate for at least 3 $\frac{1}{2}$ months without any apparent loss of infectivity.¹³

Filtration of the Leukemic Extracts Through Gradocol Membranes

In a recent series of experiments, extracts were prepared in the usual manner from leukemic Ak mice or from C3H donors with Ak-agent-induced leukemia. The 7000 \times g supernate was diluted to obtain an extract of 2.5 per cent concentration, which was passed through a Sela 02 filter candle, and then through gradocol membranes. The latter were obtained from the Wright-Fleming Institute of Microbiology, St. Mary's Hospital Medical School, London, England.

The filtrations were carried out under positive nitrogen pressure. Ten lb. of nitrogen pressure were used for membranes of 700 and 340 $m\mu$ porosity; 15 lb. for 200 $m\mu$ or less. The extracts filtered through Sela 02 filter candles were passed first through the 700 $m\mu$ porosity membrane and then through the membrane of 340 $m\mu$ porosity; the "stock filtrate" thus obtained was then passed through membranes of porosities of 200, 140, 93 or 60 $m\mu$.

* This refers to a "passage virus" developed originally from a spontaneous C58 leukemia. As this manuscript goes to press, another passage virus has been developed from spontaneous Ak leukemia that appears even more potent, inducing leukemia in from 40 to 100 per cent of the inoculated newborn C3H mice within 2 $\frac{1}{2}$ to 3 $\frac{1}{2}$ months.¹³

The filtrates were then inoculated into newborn (less than 16-hour-old) C3H or C3H/He mice. These experiments are now in progress; extracts filtered through membranes of 340 or 200 $m\mu$ porosity already have been capable of inducing typical generalized leukemia. On the other hand, extracts passed through membranes of 140 or 93 $m\mu$ porosity were still able to induce parotid tumors. Additional data are needed to evaluate these results, particularly in view of the fact that these experiments have not yet been completed.

Electron Microscopic Examination of Leukemic Extracts

In a series of experiments, leukemic extracts prepared from the leukemic Ak mice or from C3H donors with Ak-agent-induced leukemia were filtered

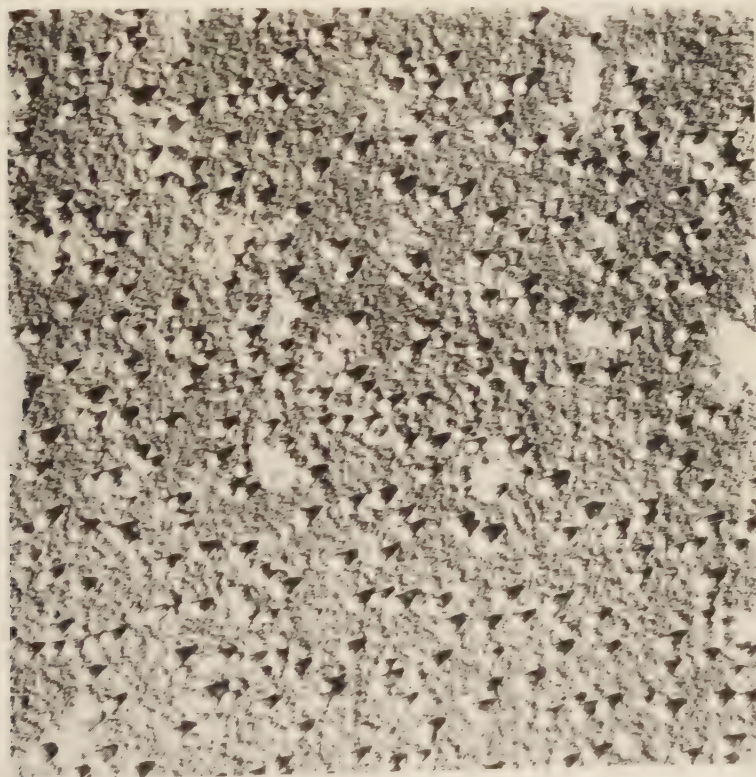


FIGURE 4. Electronmicrograph of a leukemic extract prepared from spleen, liver, and lymphoid tumors of a leukemic C3H donor in which leukemia was induced with "passage" virus. The centrifuged ($7000 \times g$) extract was filtered through Sela's porosity 02 filter candle, then through gradocol membrane porosity 740 $m\mu$, and finally through membrane of 340 $m\mu$ porosity. A drop of the final filtrate was then placed on a collodion screen, fixed in osmic acid vapor, and shadowed with chromium. The numerous spherical particles vary in diameter from 30 to 70 $m\mu$. Marvin D. Boatright aided in the electron microscopy of this sample. An RCA Electron Microscope EMU was used. $\times 33,000$

through either Selas filters only, or through Selas filters and then through gradocol membranes. A drop of the filtrate was then placed on collodium-covered screens, fixed in osmic acid vapor, shadowed with chromium, and examined in an RCA EMU electron microscope. Innumerable spherical particles varying in diameters from 30 to 70 $m\mu$ could be seen in most of the leukemic extracts examined, particularly in those obtained from leukemias induced with the high-potency-passage virus (FIGURE 4).

Discussion

Attempts to transmit mouse leukemia by filtered extracts succeeded when newborn (less than 16-hour-old) mice of a susceptible strain were used for inoculation.¹⁻³ The striking and unexpected result, however, was the development, among some of the inoculated mice, of tumors different from leukemia and rarely, if ever, occurring spontaneously in untreated mice of the strain used for inoculation (FIGURE 1). The tumors induced with the cell-free leukemic extracts were either parotid gland carcinomas⁴ or subcutaneous fibromyxosarcomas,^{6, 7, 8, 11} and occasionally medullary adrenal tumors^{6, 7} or mammary carcinomas.¹² When, in turn, cell-free extracts were then prepared from the induced leukemias, parotid tumors, or sarcomas, and then inoculated into newborn C3H mice (FIGURE 1), their pathogenic potential was highest when prepared from induced leukemias (50 per cent), substantially less when prepared from parotid tumors (33 per cent), and rather weak when made from the subcutaneous sarcomas (16 per cent). However, regardless of whether they were prepared from the induced C3H leukemias, the parotid tumors, or the subcutaneous sarcomas, such cell-free extracts, when inoculated into newborn C3H mice, were capable of inducing, although in different degree, either leukemia (7 to 37 per cent), parotid tumors (5 to 10 per cent), and/or fibromyxosarcomas (4 to 10 per cent).

The development of leukemia in some of the injected mice, and that of parotid tumors and/or subcutaneous fibromyxosarcomas in others, was quite unexpected, and it immediately raised fundamental questions which have not yet been fully answered.

There remains the basic question whether one agent inoculated into newborn C3H mice is capable of inducing either leukemia, parotid carcinomas, or subcutaneous sarcomas, or whether three or more distinct oncogenic agents are present in such extracts.

It is of considerable interest to know that mouse leukemia transferred from one mouse to another by the conventional method of cell-graft would reproduce only leukemia in the inoculated hosts. However, when cell-free (centrifuged or filtered) extracts were prepared from leukemic organs and inoculated into newborn mice of a susceptible strain, the result was not always leukemia but was, in some instances, carcinoma (of the parotid glands) or fibromyxosarcoma. Again, when such carcinomas, or sarcomas, were transplanted to other hosts by the conventional method of cell-graft, identical tumors were reproduced, that is, carcinomas or sarcomas, respectively, but when cell-free extracts were prepared from either carci-

nomas or sarcomas and were inoculated into newborn susceptible mice, in some instances at least, leukemia resulted (FIGURE 1).

Obviously, one could explain this by assuming that the same agent might have caused either disease. It is also possible to speculate, however, that a mixture of distinct oncogenic viruses was present in the leukemic extract. Mouse leukemia, or mouse carcinoma or sarcoma, may carry different oncogenic agents, even though only one of them at a time may assert itself and induce a corresponding tumor. The conventional transfer of a tumor by graft may not reveal the presence of all the potential oncogenic agents, while a filtrate prepared from a tumor and injected into a susceptible host under proper experimental conditions may reveal the presence of hitherto obscure oncogenic agents. This would explain the results obtained by Graffi *et al.*¹¹ In their experiments, filtered extracts prepared from a transplanted carcinoma or sarcoma and inoculated into newborn mice induced frequently typical leukemia.

Similarly, in recent studies, Carr¹² induced renal adenocarcinomas in young chicks inoculated with leukemic filtrate. In these experiments it was necessary to inoculate very young hosts with the cell-free extracts in order to induce renal carcinomas. This was also true in our experiments in which filtered mouse-leukemia extracts induced parotid tumors when inoculated into newborn C3H mice, but did not, with few exceptions, prove pathogenic when injected into adult animals.

Should the results of further studies be consistent with the assumption that the leukemic hosts carry different oncogenic agents, it would not be surprising to find that such agents, although possibly of a different pathogenic potential, were related.

Conclusions

Cell-free extracts prepared from "spontaneous" mouse leukemia, following inoculation into newborn mice of a susceptible strain, induced either leukemia, parotid carcinomas, or subcutaneous fibromyxosarcomas. This pathogenic activity of the leukemic extracts appears to be associated with submicroscopic particles (less than 70 $m\mu$ in diameter). These particles, presumably of a viral nature, are filterable, thermolabile, can be sedimented in an ultracentrifuge, and can readily be recovered from the leukemias or tumors that they induced.

Summary

When newborn C3H mice of the Bittner substrain were inoculated with filtered (Berkefeld N, or Selas 02 or 03) Ak leukemic extracts, 40 per cent of them developed either leukemia (28 per cent), parotid tumors (11 per cent), or subcutaneous fibromyxosarcomas (1 per cent). When cell-free extracts were then prepared from such induced C3H leukemias, parotid tumors, or sarcomas, and were inoculated into newborn C3H mice, their pathogenic potential was highest when prepared from induced leukemias (50 per cent), substantially less when prepared from parotid tumors (33 per cent), and rather weak when made from the subcutaneous sarcomas (16 per cent).

However, regardless of whether they were prepared from the induced C3H leukemias, the parotid tumors, or the subcutaneous sarcomas, such cell-free extracts, when inoculated into newborn C3H mice, were capable of inducing, although in different degree, either leukemia (7 to 37 per cent), parotid tumors (5 to 10 per cent) and/or fibromyxosarcomas (4 to 10 per cent).

Of the 70 filtered extracts prepared from different Ak donors, 18 were inactive on inoculation tests. Extracts prepared from induced C3H leukemias were more active than those from spontaneous Ak leukemias. By passing the agent serially through several successive cell-free inoculations into newborn mice, it was possible to obtain extracts of high activity. The extracts could be preserved at -70°C . for at least $3\frac{1}{2}$ months. The leukemic extracts could be inactivated by heating at 65° to 68°C . for 30 min. Exposure of the leukemic extracts, *in vitro*, to ethyl-ether destroyed their leukemogenic potential without affecting their ability to induce either parotid tumors or subcutaneous sarcomas.

When the cell-free leukemic extracts were centrifuged for 30 min. or longer in Spinco L Ultracentrifuge at 40,000 rpm, using Swinging Bucket Rotor SW 39 L (average $125,000 \times g$), the supernate was, with rare exceptions, cleared of its pathogenic activity; only the sediment pellet was then capable of inducing either leukemia, parotid tumors, or subcutaneous sarcomas.

Preliminary results of gradocol filtration experiments, still in progress, appear to suggest that the leukemogenic activity of the extracts was preserved after filtration through $370\text{ m}\mu$ and also through $200\text{ m}\mu$ membranes, whereas the ability of the extracts to induce parotid tumors was still preserved after filtration through porosity 140 and $93\text{ m}\mu$ membranes.

Electron microscopic examination of filtered leukemic extracts, particularly from donors in which leukemia was induced with the passage agent of high potency, revealed the presence of innumerable spherical particles varying in diameter from 30 to $70\text{ m}\mu$.

Acknowledgments

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LEUKEMIA OF ADULT MICE CAUSED BY A TRANSMISSIBLE AGENT*

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The attempts to transmit leukemia to adult mice with cell-free material have been numerous, but only a few have met with some measure of success. Two of these reports of successful transmission deserve mention, particularly in connection with Gross's work, included elsewhere in this monograph. Engelbreth-Holm and Frederiksen² have reported that 36 of 179 young Ak mice that had been inoculated with cell-free preparations of lymph nodes from leukemic mice of the same strain developed leukemia at an age when the spontaneous disease was rarely seen. Similar findings in the same strain of mice have recently been described by Schwartz *et al.*³ These workers, using filtrates of the brain of leukemic animals, obtained a higher percentage of "takes." Both papers pointed out the possibility that the leukemias may have been of spontaneous origin, but that they were accelerated by the treatment.

The present report concerns a disease that has a marked resemblance to leukemia and that has been found to be serially transmissible to adult mice by means of cell-free filtrates. The report stems from earlier studies on the Ehrlich ascites tumor, initiated after the electron microscopic examination of this tumor had shown that some of the cells contained cytoplasmic particles that were suggestive of virus infection.³ In the light of Morgan's findings, elsewhere in this monograph, it is easy to see how misleading such an interpretation may be. In any case, an attempt was made then to explore the possibility of the viral etiology of this tumor. Extracts of the ascites cells were made. After being centrifuged at high speed, the supernatant fluid was inoculated into infant Swiss mice. The filterable agent under discussion was recovered from a mouse in one of these experiments.

Origin of the Agent

The agent was isolated from the spleen of a 14-month-old mouse that had been inoculated in infancy with the cell-free extract of the Ehrlich tumor cells. There was no evidence of this tumor at autopsy, either grossly or histologically, but the spleen and liver were enlarged many times the normal size and appeared leukemic. Transfer of this abnormal spleen into adult Swiss mice resulted in takes in three of the five mice inoculated intraperitoneally. Three serial transfers with spleen suspensions were made before the first cell free passage was attempted. The filtrate of this extract proved to be as active as the unfiltered material in transferring the disease. This is seen clearly in the comparative titrations of the infective agent in the supernatant fluid of a suspension of spleen cells and the filtrate of the same mate-

* For full details of this work see C. Friend.¹

TABLE 1
TITER OF THE AGENT IN INFECTED SPLEEN*

Supernatant of cell homogenate			Filtrate of supernatant	
Dilution†	No. mice inoculated	No. mice positive	No. mice inoculated	No. mice positive
10 ⁻¹	5	4	5	5
10 ⁻²	5	3	5	5
10 ⁻³	5	4	5	3
10 ⁻⁴	5	2	5	0
10 ⁻⁵	5	2	5	0
LD ₅₀	10 ^{-3.4}		10 ^{-3.2}	

* Spleen from a mouse sacrificed 21 days after inoculation.

† Two-tenths ml. of each dilution, I.P.

rial. In the experiment shown in TABLE 1, the LD₅₀ of the cells was 10^{-3.4}; that of the filtrate, 10^{-3.2}.

Serial Passage

Two serial passages in adult Swiss mice are routinely maintained. One passage is made by transferring cell suspensions and the other by transferring cell-free filtrates of infected spleens. Before filtration through Sela 03 filter candles, 0.2 cc. of a 24-hour broth culture of *Escherichia coli* is added to each 10 cc. of spleen extract. To check the integrity of the filters, samples before and after filtration are inoculated into tubes of nutrient broth. Free from *E. coli*, the filtered fluid readily transmits the disease. The agent has also been found to pass through Berkefeld N filters and gradocol membranes with a pore size of 220 mμ.

TABLE 2
RESULTS OF PASSAGES
Serial Transmission of the Disease by Means of Spleen Cell Suspensions
or Cell-Free Filtrates

Material from:	Cell suspension			Cell-free filtrate		
	No. mice inoculated	No. positive	Per cent positive	No. mice inoculated	No. positive	Per cent positive
Passages 1-10.....	129	72	55.8	94	63	67.0
Passages 11-20.....	119	99	83.1	125	106	84.8
Passages 21-26.....	70	63	90.0	59	53	89.8

The results to date of the serial transmissions are presented in TABLE 2. There is a marked similarity in the number of positive animals, whether inoculated with cells or with filtrate. The increase in positive takes in the most recent passages as compared with the earlier ones might be considered as indicative of an increasing adaptation of this agent to the host, as frequently occurs with newly isolated viruses. On the other hand, since we now use only those mice weighing from 16 to 19 gm., the results may merely be due to the use of more uniformly susceptible groups of animals:

Description of the Disease

The disease that follows the inoculation of this agent is characterized by a latent period of about 2 weeks, after which the spleens become enlarged and can be felt by palpation. From then on the course is chronic. The animals do not appear ill until several days before death. The number of deaths varies with each experiment, but usually exceeds 85 per cent. The time at which death occurs after the injection of either a suspension of cells or a filtrate of the suspension is shown in FIGURE 1. There were 110 mice in each series. The range in the time of death was from 30 to 100 days. Occasionally a mouse died earlier or survived for a longer period. As was seen with the transmission experiments, the survival time was remarkably similar in both groups, being 77 days for those injected with cell suspensions

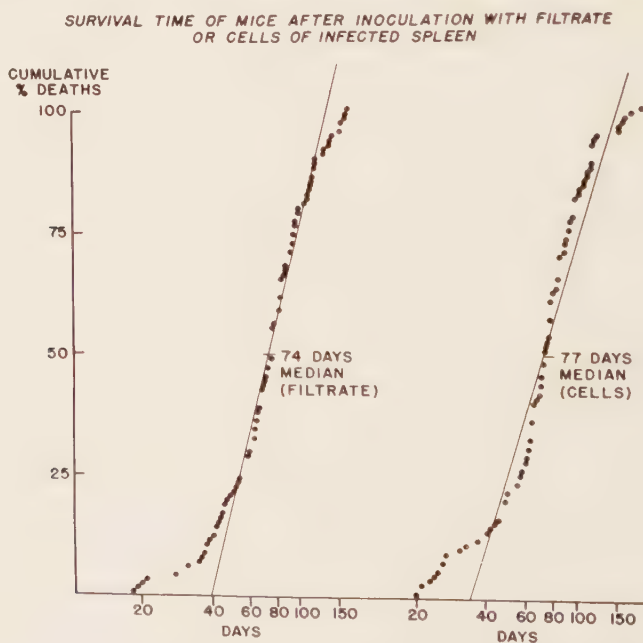


FIGURE 1. The cumulative percentage of deaths is plotted against post inoculation days to show the median survival time of 110 mice after inoculation with either the filtrate or cells of an infected spleen.

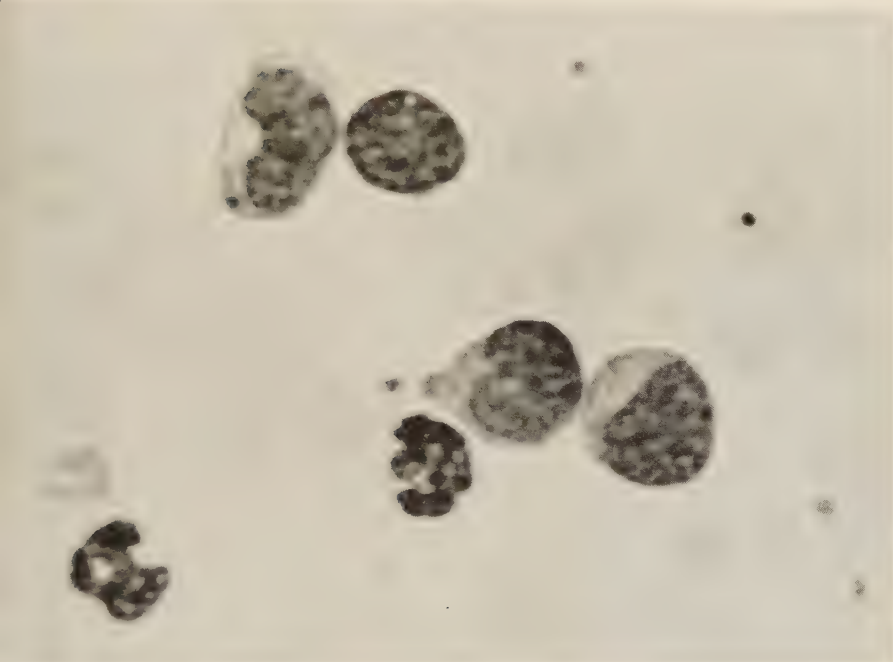


FIGURE 2. Peripheral blood smear of a mouse on the 81st day after inoculation. The WBC was 230,000 cells mm^3 , and the RBC was 4,700,000 cells mm^3 . Polymorphonuclear leukocytes, a lymphocyte, and the abnormal mononuclear cells are present. $\times 820$.

and 74 days for those injected with filtrates. The disease produced was identical in regard to the incubation period, the pathology, and the hematological changes.

The white blood count usually remains under 50,000 cells per cubic millimeter in the first few weeks after inoculation. A rise occurs between the fifth and seventh week and, terminally, may reach over 300,000. As the white count rises, there is a marked decrease in the red blood cell count. The peripheral blood contains large immature mononuclear cells, the nuclei of which may have a round, doughnut, or horseshoe shape (FIGURE 2). These cells are very fragile and, in the smears, often appear smudged. Their origin is still undetermined. In the terminal stages of the disease, nucleated red blood cells may also be found in the peripheral blood.

The characteristic gross findings in a mouse that died of the disease 57 days after inoculation appear in FIGURE 3. A normal mouse is shown for comparison. The spleen and liver are greatly enlarged. Although the normal spleen weight is 0.2 gm. or less, these spleens may weigh from 2 to 8 gm. and may often rupture. The lymph nodes are moderately enlarged, and the other organs appear normal.

The pathological changes that take place in the organs of these mice are striking. The bone marrow, spleen, and liver are infiltrated with tre-

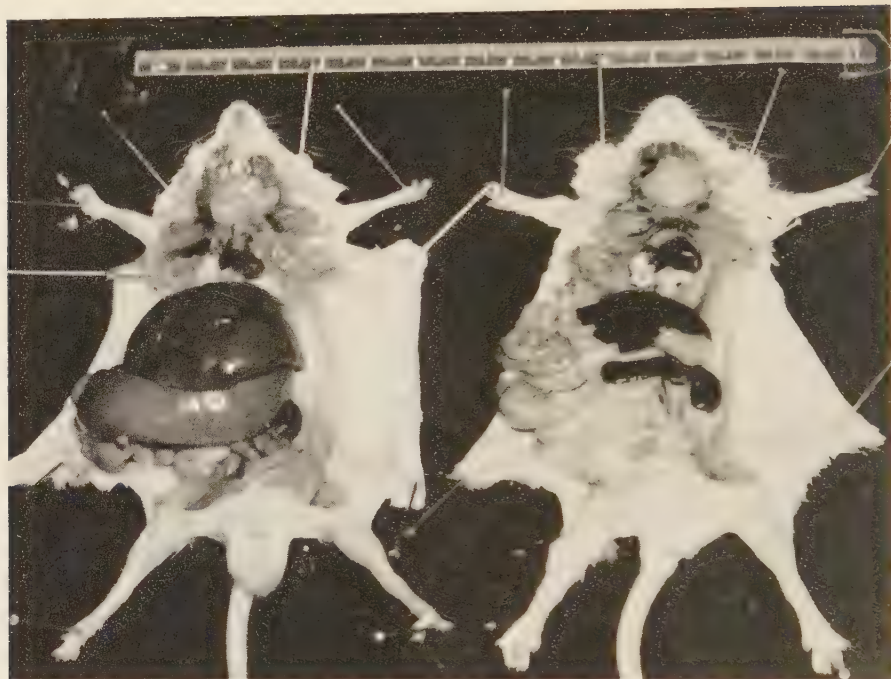


FIGURE 3. Marked enlargement of the liver and spleen in the mouse on the left, which was found dead 57 days after inoculation. The mouse on the right is an untreated control of the same age.

mendous numbers of abnormal cells. These cells are relatively large and mononuclear. They have round pale nuclei and thin rims of cytoplasm. A decalcified section of the sternal marrow from a mouse in the terminal stage of the disease is shown in FIGURE 4; the abnormal cells have replaced a major portion of the normal marrow cells. In the spleen these cells are found in both the follicles and in the red pulp. In nearly all instances of severe involvement, the demarcation of the follicles and the pulp is lost, and almost the entire spleen is replaced (FIGURE 5). The liver is involved in a similar manner (FIGURES 6, 7). The sinusoids are dilated by immense numbers of the large mononuclear cells, and there is marked atrophy of the hepatic cords. In some areas, because of the extensive infiltration, the organ could hardly be recognized.

Renal involvement is minimal, and occasional focal collections of the infiltrating cells are seen. In the lung the septa are thickened and distended, and the alveolar spaces are diminished in size. Infiltration of the lymph nodes is moderate.

Routes of Inoculation

The mice can be infected by intraperitoneal, subcutaneous, intracerebral, and intramuscular routes. In mice inoculated by the latter three routes,

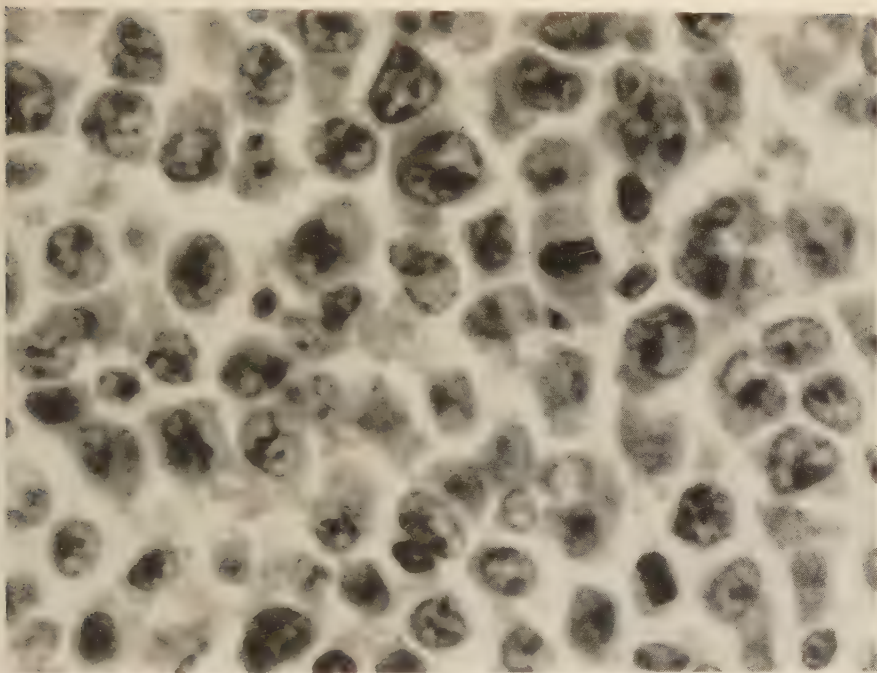


FIGURE 4. A section of decalcified sternal marrow of a mouse 88 days after inoculation. There are many large mononuclear cells with vesicular nuclei. Mitotic figures are present. $\times 920$.

the onset of the disease may be delayed several days, as compared with those injected intraperitoneally, but the course remains essentially the same. No tumor forms at the site of inoculation, but the generalized leukemia-like disease develops. This is not unique, since local growths in some mouse leukemias may be small or absent.

Transmission with Tissues Other Than Spleen

It is also possible to transmit the disease with the cells from organs other than the spleen of infected mice. This is shown by the data presented in TABLE 3. The organs tested were from mice sacrificed 16 days after inoculation. A 10 per cent suspension was made from each organ pool, but the blood was inoculated undiluted. Preparations from the kidney and spleen, as well as the blood, transmitted the disease to all the injected animals. The suspensions from the liver, lung, and node were 70 to 80 per cent positive and those from the brain, heart, and skin ranged from 40 to 50 per cent. These results are of interest only in that they show the distribution of infective material in various tissues, since the supernatants of the suspensions and not the filtrates were tested.

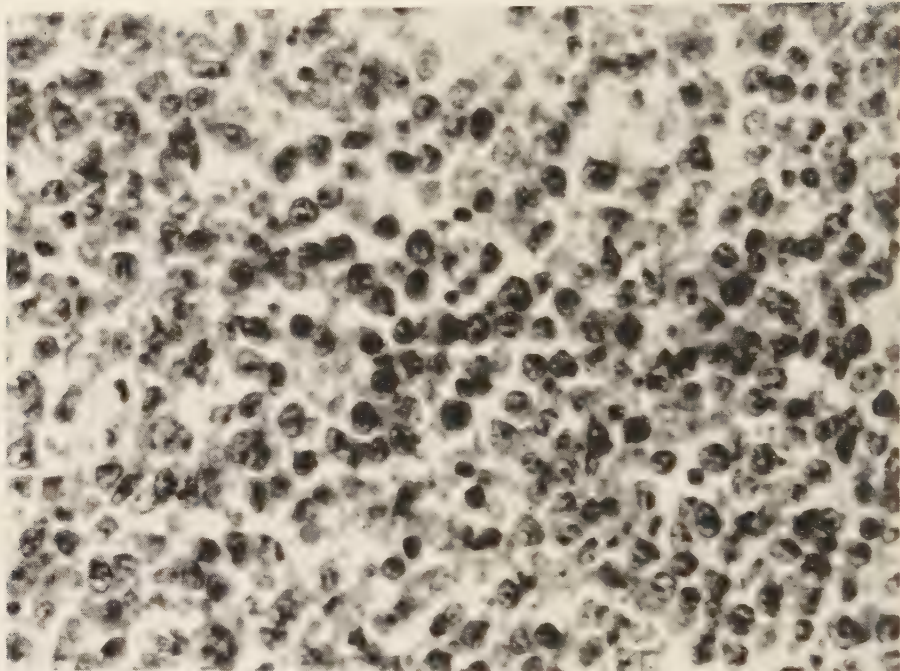


FIGURE 5. The spleen, showing loss of demarcation of the follicles and the red pulp. $\times 400$.

Strain Specificity

Since the agent was isolated from a Swiss mouse, this has been the strain chiefly used in these studies. A number of adult mice of other strains, however, have been examined for susceptibility to infection with the agent.

TABLE 3
THE TRANSMISSION OF THE DISEASE BY THE INOCULATION OF HOMOGENATES OF VARIOUS ORGANS OBTAINED FROM INFECTED MICE*

Organ†	No. inoculated	No. positive	Per cent positive
Spleen.....	10	10	100
Whole blood.....	8	8	100
Kidney.....	10	10	100
Lymph nodes.....	10	8	80
Lung.....	10	8	80
Liver.....	10	7	70
Heart.....	4	2	50
Brain.....	10	4	40
Skin.....	5	2	40

* Sacrificed 16 days after inoculation.
† Two-tenth ml. I.P. of supernatant of 10 per cent homogenate (blood undiluted).

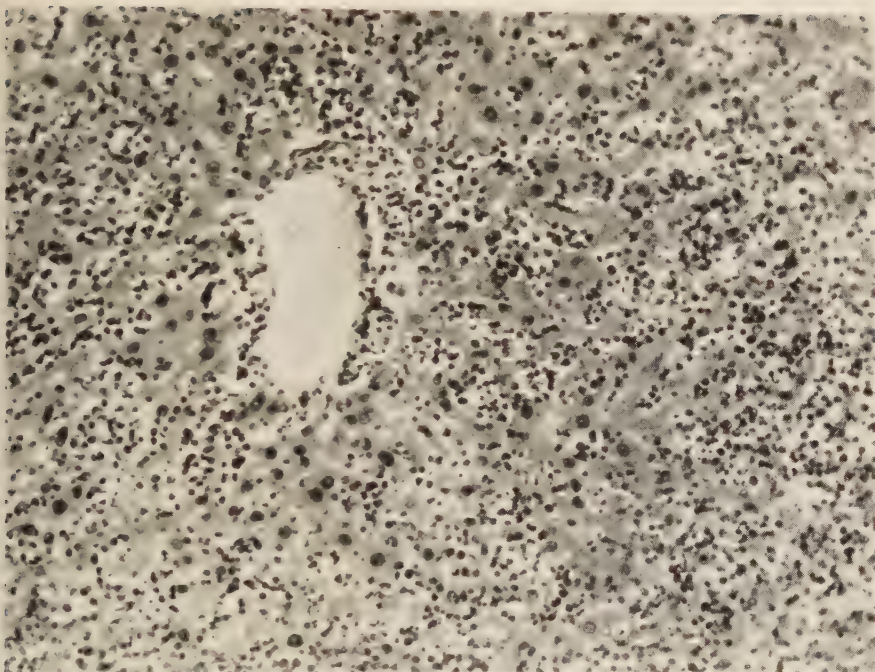


FIGURE 6. Liver, showing massive infiltration. The sinusoids are distended by large numbers of the mononuclear cells. $\times 112$.

Other than the Swiss, thus far, only the DBA/2 have proved susceptible; eighteen serial transfers have been carried in this line. The PRI, C57B1/6, A, C3H, and F1(C58 \times BALB) are resistant to the disease.

Stability of the Agent

Some experiments have also been carried out to determine the stability of the agent to physical and chemical treatment (TABLE 4). The agent has

TABLE 4
STABILITY OF THE AGENT

Treatment	Material	No. inoculated	No. positive	Per cent positive
X ray (50,000 r).....	Whole spleen	20	17	85
Lyophilized (3 mo.).....	Filtrate	10	10	100
4° C. (11 days).....	Filtrate	10	7	70
-70° C. (6 mo.).....	Filtrate	10	6	60
-70° C. (1 yr.).....	Filtrate	10	8	80
56° C. (30 min.).....	Filtrate	10	0	0
Ether (overnight, 4° C.).....	Filtrate	15	0	0
Formalin (1:200).....	Filtrate	20	0	0

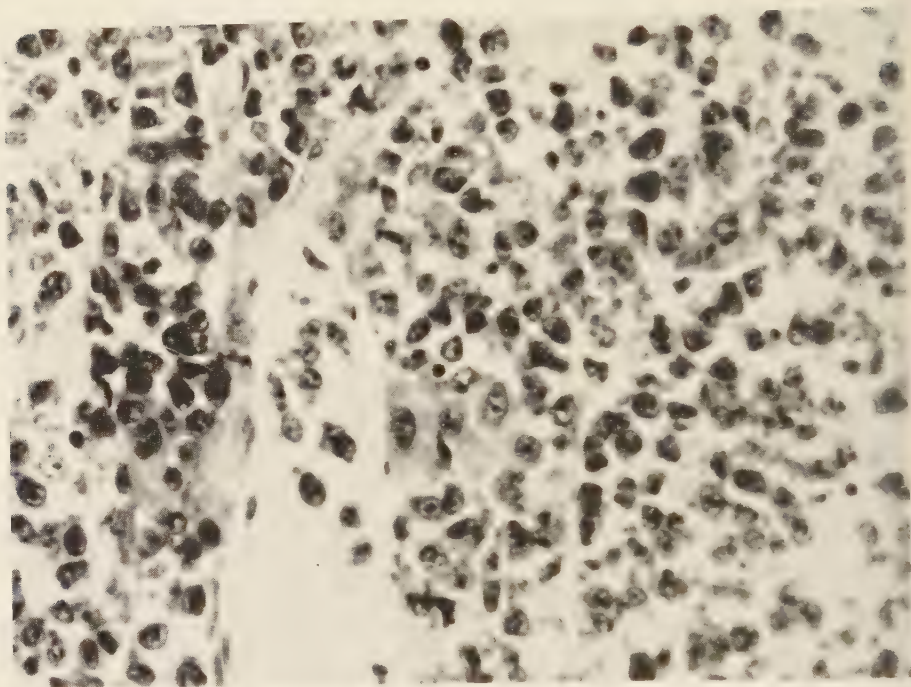


FIGURE 7. Another area of the same liver section as shown in FIGURE 6, at higher magnification, showing atrophy of the liver cords. Some cells lie within the blood vessel. $\times 400$.

shown marked resistance to massive doses of X ray. After *in vitro* exposure to 50,000 r, there is no difference in the infectivity of the irradiated infected spleen as compared with the nonirradiated sample of the same spleen. Experiments to find the limits of resistance to X irradiation are in progress, and they indicate that spleen fragments exposed to 100,000 r and 150,000 r still retain infectivity.

The pooled results of two such experiments are given in TABLE 4, along with other data on the stability of the agent. Lyophilized filtrates retain their infectivity after storage for 3 months. Filtrates of infected spleen are still active after having been stored at -70°C . for 1 year, and for at least 11 days at 4°C .

The activity is destroyed by heating at 56°C . for 30 min., by overnight exposure to ether at 4°C ., and by treatment with a 1:200 dilution of formalin.

Relationship of the Agent to a Known Virus

In view of the experiences of Stewart and Haas⁵ and of the many virologists who have encountered lymphocytic choriomeningitis (LCM) as a latent virus in their experimental animals, it seemed desirable to find out whether there was any simple immunological relationship between this virus and the agent under discussion. This was done despite the fact that the disease

TABLE 5
LACK OF IMMUNOLOGICAL RELATIONSHIP BETWEEN THE AGENT AND LCM
A. Neutralization Test*

	Normal serum		Anti-LCM serum	
	No. deaths/ No. inoculated	Per cent positive	No. deaths/ No. inoculated	Per cent positive
Agent	20/20	100	18/19	94.7
LCM	20/20	100	0/20	0

* Three-hundredths ml. I.C. of serum-virus mixture after incubation at 37° C. for 1½ hr

B. Protection Test

Inoculum	Control mice		LCM-immunized mice	
	No. deaths/ No. inoculated	Per cent positive	No. deaths/ No. inoculated	Per cent positive
Agent*	14/15	93.3	17/19	89.6
LCM†	20/20	100	0/19	0

* Three hundredths ml. I.C. of 10⁻¹ dilution of spleen filtrate.

† Three hundredths ml. I.C. of 10⁻² dilution of LCM-infected brain.

produced by the agent was completely different from that observed in LCM infected mice.

The results of neutralization and protection tests presented in TABLE 5 indicate that the agent and LCM are not related. Mouse anti LCM serum failed to neutralize the agent, while it completely neutralized the Armstrong strain of the LCM virus (TABLE 5A). The protection tests confirmed the findings of the neutralization tests. LCM-immune mice were fully susceptible to the agent, but resisted LCM infection (TABLE 5B).

Conclusion

This report has summarized our present knowledge about the agent and the disease it causes. Many questions remain to be answered but, in conclusion, only three of them will be raised.

First, is this disease a true leukemia? It is fatal and can be transmitted serially in adult mice. The pathological changes are characteristic of the leukemias rather than of simple metaplasia,⁶ which has never been shown to be transmissible. While there are erythropoietic elements found in the involved organs, as is demonstrated by the presence of nucleated red cells, the abnormal mononuclear cells typical of those associated with leukemia are much more prominent. There is uncontrolled proliferation of these cells;

they invade the spleen, liver, bone marrow, kidney, and lung, and they also appear in the peripheral blood. Terminally, the mice have greatly elevated white blood counts, are anemic, and have tremendously enlarged spleens and livers.

Second, what is the nature of the agent producing this disease? Its viral character is indicated by the fact that (1) it can be transmitted by filtrates prepared under conditions in which all cells of the size of *E. coli* are held back; (2) it is resistant to massive doses of X ray, more than sufficient to destroy mammalian cells; and (3) it is stable for long periods of time in the dried or frozen state.

Third, what is the source of the agent? Both Graffi and his co-workers⁷ and Schmidt⁸ have reported that cell-free preparations from the Ehrlich tumor will induce leukemia when inoculated into newborn Agnes-Bluhm mice. Their findings, however, differ from those presented here in at least three respects: (1) this agent has a much shorter period of latency; (2) it produces the disease in adult mice; and (3) it can be passed serially in such animals.

Although the agent we are considering was initially recovered from a mouse that had been inoculated in infancy with material from the Ehrlich ascites tumor, it is still not clear whether it was latent in the tumor or whether its relationship to the tumor was accidental. The possibility that the agent was the cause of a spontaneous disease that developed in this animal some time during the fourteen month observation period should be considered.

Regardless of its origin, however, the fact remains that a filterable agent that consistently produces a leukemia-like disease in adult mice is available.

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STRAIN SPECIFICITIES OF LEUKEMIA AGENT*

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Strain specificity evaluations have been secured from three groups of experiments: (1) cortisone studies using high- and low-incidence leukemia mice;¹⁻³ (2) experiments on cell-free transmission of mouse leukemia;^{4, 5} and (3) studies with a new leukemia of viral origin.⁶ Strain-specificity data have, for the most part, been incidental in these problems. It seems probable that all three areas are concerned with the operation of subcellular particles in the neoplastic process.

Chronic Treatment with Cortisone

High-incidence leukemia mice. We have observed that chronic administration of cortisone to high-leukemic strain AKR mice significantly lengthened the time of occurrence of leukemia. The incidence of leukemia was also reduced. In extending this work to other high-leukemia strains we unexpectedly observed the occurrence of parotid gland tumors in the cortisone-treated and not in the control mice. These tumors were of special interest to us, not only because they were a rare type of tumor and apparently cortisone-induced, but also because they were similar to the parotid gland tumors observed by Gross⁷ in C3H mice treated when newborn with cell-free material prepared from high-leukemic strain tissues.

In our study, cortisone acetate† was administered (1 mg., day subcutaneously) in divided doses as a saline suspension, 3 successive days once each month throughout life, starting when the mice were 1 month of age. The mice were of strain AKR (Bar Harbor), C58, and of the 5th to the 10th generation of a new strain we are developing following a cross between strains C58 and AKR. All were high-incidence leukemia strains. Control mice were untreated and from the same parental stocks. Males and females were in approximately equal numbers.

Strain AKR mice failed to develop parotid tumors following cortisone treatment. In strains C58 and C58 \times AKR there was a total of 6 parotid tumors, 2 in strain C58 and 4 in C58 \times AKR (3.5 and 8.0 per cent, respectively). The tumors occurred in the males as well as the females of each strain. The mean age at autopsy of the animals with this type of tumor was 11 months. The tumors were slow-growing and, as a rule, were observed for a few months before autopsy. Two of the tumors were transplanted, one successfully. No parotid gland tumors were observed in the control mice.

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† Merck Sharp & Dohme, Philadelphia, Pa.

Low-Incidence Leukemia Mice

In view of the above observations, the possibility of cortisone treatment leading to parotid tumor formation in a low-incidence leukemia strain of mice such as strain C3H was considered. It has been well established, following the work of Gross,⁷ that parotid tumors may develop in C3H mice as a result of treatment when newborn with cell-free extracts of leukemia tissues.⁸⁻¹⁰ Extracts prepared from other than leukemic tissues also had some influence.¹¹ Would the various sublines of C3H be similar or different in their response to cortisone? The test mice were from two sublines of strain C3H. One was a fostered subline secured from Gross, and is here designated C3H_i Gs. This was expanded in our laboratory from a small start of pedigreed mice. Another subline was secured from the Jackson Memorial Laboratory, Bar Harbor, Me., and is known as Hummel's fostered subline, C3H_i Hu. The former was developed from the Bittner subline and the latter from the Andervont subline.

Cortisone treatment was given twice daily (1 mg. day subcutaneously) as a saline suspension, 3 successive days once each month. Treatment was started when the mice were 1 month of age, and was continued throughout life. The controls were untreated mice of the above 2 sublines developed from the same parental stocks.

Four parotid tumors occurred in 42 cortisone-treated C3H_i Gs mice (9.3 per cent) at a mean age of 12.5 months. The average age of dead nontumorous mice was 13.0 months. No parotid tumors appeared in 46 treated C3H_i Hu mice observed to a mean age of 13 months, nor in 55 control mice autopsied at advanced ages. The results are summarized in TABLE 1.

As far as parotid gland tumor occurrence is concerned, the results suggest a subline difference in response to cortisone. Evidence has been presented indicating that the Andervont subline, related to C3H_i Hu, is relatively resistant to leukemia following inoculation with cell-free filtrates prepared from leukemic tissue. It would be of interest to determine further whether this subline is also resistant to the formation of tumors of the parotid gland. Other C3H sublines and also other strains of mice could well be tested with chronic administration of cortisone to determine the type and number of

TABLE 1
PAROTID GLAND TUMORS IN CORTISONE-TREATED AND CONTROL STRAIN C3H MICE

Cortisone-treated C3H	No. treated	Parotid tumors		Mean age, tumor	No. dead, non-tumor	Age non-tumor dead	Age alive
		No.	Per cent				
C3H _i /Gs.....	42	4	9.3	12.5	38	13.0	—
C3H _i /Hu.....	46	0	0	—	46	13.0	—
Controls C3H _i /Gs.....	22	0	0	—	19	18.0	26.0
C3H _i /Hu.....	33	0	0	—	31	20.5	26.0

tumors that arise. It would also be of interest to test mice inoculated when newborn with cell-free extracts prepared from leukemias, parotid gland tumors, and other forms of cancer.

Law *et al.*⁹ have called attention to litter susceptibility to parotid gland tumor formation. This did not seem to be pronounced in our study. Two of the four individuals with parotid tumors were from the same parents, but from different litters. The other two were from other and independent parent pairs and were only distantly related to the former two individuals. A number of sibs of each tumor-bearing individual were present in the experiment without developing parotid tumors.

It is concluded that chronic treatment of mice with cortisone offers promise of bringing out hidden potential cancer tendencies of different strains of mice. Chronic cortisone treatment may be of importance in activating as well as in inhibiting certain subcellular particles that are of importance in cancer.

Experiments on Cell-Free Transmission of Mouse Leukemia

We have been investigating the relationship of cell-free extracts prepared from leukemic tissues—centrifugates and filtrates—to the induction of cancer, particularly leukemia in mice.

These studies were started because of the interesting observations made by Gross that cell-free, viruslike material prepared from either leukemic tissue or normal tissue from leukemic strain mice would, when given to newborn mice of different susceptible strains, induce leukemia and other forms of cancer.^{7, 11}

The strains of mice used in this study were Akn Gs (Gross), C3H⁺/Cs, C3H_i Gs (i signifies fostered to eliminate the viruslike milk factor), C3H_i/Bi (Bittner), C3H_i Hu (Hummel), and C3H Wy (Woolley). The extracts were inoculated into newborn mice, less than 12 hours old in most instances, frequently less than 6, and never more than 16. Following weaning, the mice were carefully examined at weekly intervals for tumor formation. The control mice were obtained from alternate litters of the same parental mice as were the treated individuals, and were maintained as nonbreeders in small groups of 5 or 6.

The methods of preparation of the extracts and injection into newborn mice have followed closely those developed by Gross.¹¹ Twenty-per cent pooled preparations of ground thymus, liver, spleen, and lymphoid tumors were prepared with chilled (4° C.) physiological saline, centrifuged at 3000 rpm (1800 × g) for 15 min. and then at 9500 rpm (7000 × g) for 10 min. at 0° C. The final supernatant (centrifugate) was used for inoculation or was passed through a Selas 02 or 03 porosity filter under a vacuum pressure of 20 to 25 mm. of mercury (filtrate) prior to use. Prior to filtration, the centrifuged extract (10 cc.) was mixed with a diluted broth culture of *Escherichia coli* (0.5 cc.). As evidenced by inoculation of ordinary culture media, the filtrates were bacteriologically sterile, so it may thus be assumed that these filtrates were cell-free. All extracts were kept close to 0° C. in ice water, and were injected (0.05 cc.) subcutaneously within 48 hours after preparation.

TABLE 2
RESULTS OF INOCULATION OF LEUKEMIC EXTRACTS INTO NEWBORN C3H MICE OF VARIOUS SUBSTRAINS

	No. treated	No. leuke- mias	No. paro- tid tumors	No. sar- comas
C3H _t /Gs.....	189	39	7	9
C3H _t /Bi.....	17	5	4	3
C3H/Gs.....	30	3	0	1
C3H _t /Hu.....	63	7	0	0
C3H/Wy.....	21	0	0	0
Total.....	320	54	11	13

Portions of the tumors that developed were fixed in Vandergriff's fluid and were sectioned and studied histologically. Liver sections were secured for aid in determining leukemia. Many of these tumors were transferred by trocar or cell-suspension to mice of both the strain of extract and the strain of tumor origin for analysis of genetic status.

Preliminary results have been summarized recently.⁵ A more recent summary of the data by strain is shown in TABLE 2. Of 320 strain-C3H mice observed, 54 have exhibited leukemia, 11 parotid gland tumors, and 13 subcutaneous sarcomas. The majority of these were in the C3H_t/Gs and C3H_t/Bi mice (both fostered mice of the Bittner subline). A number of leukemias have also appeared in the C3H_t/Hu mice. These were entirely from two generations of the same preparation line that originated from a C58 rather than an AKn leukemia.

Tumor transplantation data for leukemias, parotid gland tumors, and sarcomas originating in this series were secured. The tumors were specific for the host in which they arose (TABLE 3). Exceptions were with a leukemia that arose in a C3H_t/Hu mouse and was transplanted to two C3H_t/Gs mice (extract No. 3410); a C3H_t/Gs leukemia transplanted to a C3H/Wy mouse (extract No. 3426); and a C3H_t/Gs leukemia transplanted to one out of five AKn mice (extract No. 3395).

It was concluded that, following inoculations with cell-free extracts of leukemic tissue, subline differences in the susceptibility of mice to cancer may occur. Fostered mice of the Bittner sublines C3H_t/Gs and C3H_t/Bi appeared to be most susceptible.

A New Leukemia of Viral Origin

Charlotte Friend, of this Institute, has recently called attention to a new leukemia of viral origin first obtained in Swiss mice following inoculation of these animals when newborn with a cell-free extract prepared from an ascitic form of Ehrlich's carcinoma.⁶ It appears to be essentially a granulocytic

TABLE 3
EXPERIMENTS IN CELL-FREE TRANSMISSION OF MOUSE LEUKEMIA
Results of Cell Transfer from Induced Tumor to Various Strains and Substrains

Extract	Strain of extract origin	Tumor type	Strain of tumor origin	Trans- planted strain	No. tumors trans- planted	No. trans- plants	No. posi- tive	No. nega- tive
3410	C3H/Gs	L	C3H _f /Hu	C3H _f /Hu	3	4	4	0
3410	C3H/Gs	L	C3H _f /Hu	C3H/Wy	2	3	3	0
3410	C3H/Gs	L	C3H _f /Hu	C3H _f /Gs	2	4	2	2
3410	C3H/Gs	L	C3H _f /Gs	C3H _f /Gs	2	4	4	0
3410	C3H/Gs	L	C3H _f /Gs	C3H _f /Hu	1	2	0	2
3462	AKn	S	C3H _f /Bi	AKn	1	2	0	2
3462	AKn	S	C3H _f /Bi	C3H _f /Gs	1	2	1	1
3462	AKn	S	C3H _f /Bi	C3H _f /Bi	2	4	1	3
3462	AKn	S	C3H _f /Gs	C3H _f /Gs	1	3	3	0
3426	AKn	L	C3H _f /Gs	AKn	2	4	0	4
3426	AKn	L	C3H _f /Gs	C3H _f /Gs	3	5	4	1
3426	AKn	L	C3H _f /Gs	C3H/Wy	1	1	1	0
3267	AKn	L	C3H _f /Gs	AKn	1	2	0	2
3267	AKn	L	C3H _f /Gs	C3H _f /Gs	1	2	2	0
3395	AKn	L	C3H _f /Gs	AKn	3	6	1	5
3395	AKn	L	C3H _f /Gs	C3H _f /Gs	3	5	5	0
3395	AKn	L	C3H _f /Gs	C3H _f /Bi	1	2	2	0
3395	AKn	L	C3H _f /Gs	C3H _f /Wy	1	2	0	2
3333	AKn	L	C3H _f /Gs	AKn	2	5	0	5
3333	AKn	L	C3H _f /Gs	C3H _f /Gs	2	4	4	0
3450	AKn	L	C3H _f /Gs	AKn	1	2	0	2
3450	AKn	L	C3H _f /Gs	C3H _f /Gs	1	1	1	0
3651	C3H _f /Bi	L	AKn	AKn	2	4	4	0
3651	C3H _f /Bi	L	AKn	C3H _f /Bi	1	2	0	2
3400	C3H/Gs	S	C3H/Gs	C3H/Gs	1	4	3	1
3400	C3H/Gs	S	C3H _f /Gs	C3H _f /Gs	6	11	9	2
3400	C3H/Gs	S	C3H _f /Gs	AKn	1	2	0	2
3400	C3H/Gs	S	C3H _f /Gs	C3H/Gs	1	2	1	1
3400	C3H/Gs	P	C3H _f /Gs	C3H _f /Gs	6	11	7	4
3400	C3H/Gs	P	C3H _f /Gs	AKn	1	2	0	2
3498	C3H _f /Gs	L	C3H _f /Gs	AKn	4	5	0	5
3498	C3H _f /Gs	L	C3H _f /Bi	AKn	2	4	0	4
3498	C3H _f /Gs	L	C3H _f /Gs	C3H _f /Gs	9	17	17	0
3498	C3H _f /Gs	L	C3H _f /Bi	C3H _f /Gs	3	6	6	0
3498	C3H _f /Gs	L	C3H _f /Gs	C3H _f /Bi	1	2	2	0
3498	C3H _f /Gs	L	C3H _f /Bi	C3H _f /Bi	5	9	9	0
3498	C3H _f /Gs	L	C3H _f /Gs	C3H/Gs	1	1	1	0

leukemia. Passage by means of cells or cell-free filtrates can be obtained adult-to-adult in Swiss mice, but not in a number of other strains. Some successful transfers have been made to strain DBA mice and to brother-sister inbred mice following a cross of strains R111 and DBA. Strain C3H mice have been uniformly resistant to the transfer.

We have observed that, if cell-free material prepared from spleens, livers,

and lymphoid organs by the method of Gross is put into newborn rather than adult C3H mice, the leukemia will develop by the time the young are of weaning age. Following development in these young mice, the disease can be transferred adult-to-adult in C3H mice. It can also be returned readily to adult Swiss mice.

It is concluded that, although the leukemia can be transferred to certain strains through transfer of cell-free filtrates to the young, the essential strain specificity of the leukemia has not been shifted completely to the new strain.

Summary

Chronic treatment of mice with cortisone was followed by the occurrence of parotid gland tumors. These tumors, rare in mice, did not occur in the control groups. The fact that occurrence was not uniform in all the treated groups indicated possible strain or subline differences.

In experiments on the cell-free transmission of leukemia, based on the technique of inoculation of newborn mice, suggestive subline differences in susceptibility were observed. Fostered mice of the Bittner sublines, C3H_f Gs and C3H_f Bi, were most susceptible. Leukemias occurred in a fostered Andervont subline, C3H_f Hu, with only one extract. Tumor transplantation data indicate that the tumors tend to be specific for the strain in which they are induced when cell transfer is made from these tumors.

A new leukemia of viral origin has been under study. It has been observed that, although it can be transferred to a few strains beyond that of origin, one strain, C3H, is resistant. Transfer to strain C3H can be made via the newborn. Specificity for transfer to the original strain is not lost through growth in strain C3H.

It is evident that strain specificity of both agent and host play interesting and important roles, and that they should be considered in experimental procedures that relate to subcellular particles important in neoplastic processes.

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CHLOROLEUKEMIA OF MICE

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These studies were instigated by the hypothesis, proposed by a number of scientists, that a "tumor agent" that is separable from the cell is responsible for the formation of malignant tumors. For the time being it is irrelevant whether such an agent should be regarded as an exogenous virus¹⁻⁵ or as an endogenous factor originating from the cell.⁶⁻¹⁰

Further inducement to the present studies were the results of Gross's work¹¹⁻¹⁵ concerning the cell-free transfer of lymphatic leukemia of the mouse, and our own experiments with the supernatant obtained by centrifuging Ehrlich ascites tumors.^{16, 17} These latter experiments have given ambiguous results.

Methods

The following transplantable mouse tumors were effective in the production of chloroleukemia: sarcoma I and sarcoma II (Landschütz), reticulum-cell sarcomas that also grow as ascites tumors; the Ehrlich carcinoma, in ascites form and in solid form; SOV 16, a myeloid leukosis that grows locally either intramuscularly or subcutaneously (this tumor arose as a localized intrathoracic leukemia following the injection of a cell-free filtrate of sarcoma I); and sarcoma 37.

In general, the tumor filtrates were prepared in the following manner:^{18, 19}

The tumor tissues or the sedimented ascites tumor cells (sarcoma I, Ehrlich carcinoma) were homogenized at 10 to 12,000 rpm. The homogenate was usually diluted 1:10 with physiological salt solution and centrifuged at 2500 rpm (450 × g). The supernatant was usually filtered twice through a G4-Schott glass filter. In every experiment these filters were tested for their impermeability to yeast cells (2 to 3 μ in diameter). In a model experiment they proved to be impermeable to ascites tumor cells. Moreover, the filtrates were checked microscopically. All experiments were conducted rapidly at 0° C. with the exclusion of oxygen.¹⁶ In many instances cysteine was added to ensure anaerobiosis. The experimental animals were sucklings and adult mice of the inbred strains Agnes-Bluhm ("weiss"), sg ("schwarzgelb"), and db ("hellbraun")* and also noninbred animals of the strains M and W. Sucklings, usually 1 to 3 days old, were injected subcutaneously with 0.1 ml. of filtrate. The adults were injected subcutaneously, intraperitoneally, and intravenously with 1 to 4 ml. of filtrate.

Results

In order to assess correctly the experiments concerned with cell-free tumor transfer it was absolutely necessary to know the incidence of spontaneous

* The Agnes Bluhm are similar to albino strains; the "schwarzgelb" is similar to sooty yellow; and the "hellbraun" is similar to dilute brown.

tumors in the animals employed. My associates and I were especially interested in the incidence of spontaneous leukemia. We dissected 2,331 animals of the various mouse strains used in our filtrate experiments and we found only 20 leukemias, that is, an incidence of 0.9 per cent (at the time of their death none of the mice were older than 6 months). The highest incidence of spontaneous leukemia was 1.3 per cent. The average latent period of these leukemias was 479 days. No chloroleukemias were observed.

In TABLE 1 the experiments with G4 filtrates of the tumors sarcoma I, sarcoma II, SOV 16, and the Ehrlich carcinoma of the mouse are recorded.¹⁹⁻²³ In these experiments newborn animals of the Agnes-Bluhm strains M and W were treated with 0.1 ml. of filtrate. In all series leukemia appeared after a latent period of several months, and the animals died. The majority of leukemias were chloroleukemias (FIGURE 1). As compared with the untreated controls, the following differences were seen: (1) at least a fiftyfold increase of leukemia incidence; (2) an appreciably shorter latent period; and (3) a high incidence of chloroleukemia that was completely absent in the controls.

Thus far we have recorded a total of about 1000 leukemias produced by injection of cell-free filtrates of the 5 mouse tumors named above. In most instances the leukemias were generalized: lymph nodes, spleen, thymus gland, and liver were considerably enlarged (FIGURE 2). The cervical lymph nodes, the lymphatic tissues of the mesentery and, in certain instances, (tumor SOV 16) the thymus gland, showed the greatest increase of weight. In extreme cases the increase in weight of the lymphatic tissues, not including the spleen, was 50 to 100 times that of the normal. The leukemias were partly of the acute and partly of the chronic type. Hematologically, the point in question is whether chloroleukemias as well as the white forms and the yellow transition forms are myeloid leukemias (positive peroxidase reaction).^{19, 21, 24} The number of leukocytes in the blood smear was usually greatly increased (up to 300,000/ml.). In particular, however, the intra-thoracically localized leukoses (frequently after application of SOV-16 filtrates) often took an aleukemic course. A strong shift to the left of the

TABLE 1
FREQUENCY OF LEUKEMIA AFTER THE INJECTION OF CELL-FREE G4
FILTRATES OF DIFFERENT TUMORS OF MICE

Tumor	No. of sur- viving animals	No. of leukemias	Fre- quency of leukemias (per cent)	Average latent period (days)	Percent- age of chloro- leukemias
Sa I.....	145	116	80	224	67
Sa II.....	13	10	~77	255	67
SOV 16.....	20	17	~85	124	37
Ehrlich ascites carcinoma.....	71	42	59	236	67
Ehrlich carcinoma, solid form...	66	33	50	320	55



FIGURE 1.

myeloid cells was observed in the blood stream as well as in the bone marrow (FIGURE 3). Hematologically, one can distinguish immature forms by a predominance of promyeloblasts and mature forms by a predominance of neutrophilic leukocytes.²⁴

Based on our cytological investigations of the blood and the bone marrow at various intervals following the filtrate injection, we believe that the leukemogenic agent of the filtrate attacks first the myeloid cells of the bone marrow. The cells that have become malignant settle in the lymphatic nodes, the spleen, the thymus gland, and the liver, where they multiply, causing enlargement of these organs and destruction of the organ tissues. One finds extensive myeloid infiltration in the final states of the disease in the liver and in other organs, such as the lungs, the kidneys, and the ovaries.^{19, 21, 24} The leukemogenic factor is also found in the leukemic lymph nodes.²⁵ Homogenates of leukemic lymph nodes centrifuged and filtered twice through G4 filters were injected into newborn mice. In 12 surviving animals, 10 leukemias appeared after a relatively short latent period. We received the impression that the efficacy of the agent was even increased by the cell-free passage. Our findings with the SOV-16 tumor, derived from a "filtrate leukemia," and of a short latent period, led to the same results.



FIGURE 2. Chloroleukemia 118 days after injection of G4 filtrates of tumor SOV 16. The filtrate dilution is 1:1000; total weight of the lymphatic glands, >10 gm.

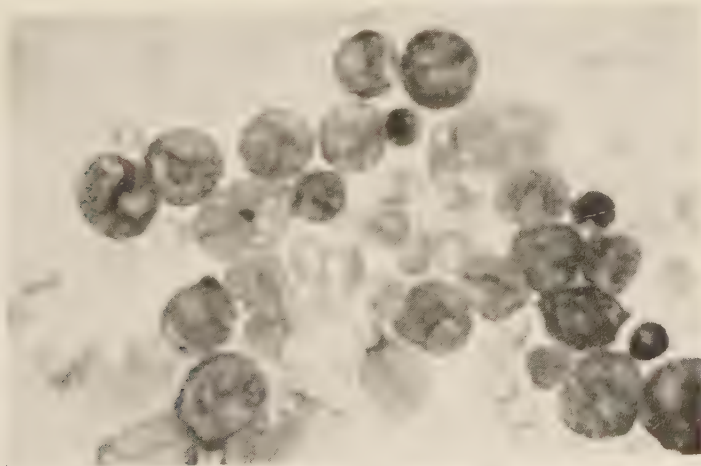


FIGURE 3. Blood smear of a leukemia after the injection of Sa I filtrate—mostly immature myeloid cells. Giemsa stain.

Transplantability

The biological property of transplantability is important in the characterization of the filtrate-produced leukemias as a neoplastic process.^{19, 26, 27} The cellular transplantation in which several millions of cells were used took, in the first passage, in about 10 per cent of the surviving animals (25 positive results in 259 animals). A serial transplantation was successful in only 4 instances. For these experiments we employed sucklings and adult animals of the same strains that were used in the filtrate experiments. Altogether, therefore, the transplantability of leukemias produced by filtrates was relatively poor. Nevertheless, this result may suffice to show the neoplastic character of these leukemias. It should be remembered also that mouse leukemias produced by carcinogenic substances lend themselves, in general, very poorly to transplantation. The fact that cellular transplantation gave much poorer results than had been obtained with G4 filtrates (10 per cent as compared with 40 to 80 per cent) would exclude whole cells as participants in our filtrate experiments. On the other hand, the original tumors used for the preparation of the filtrates were practically 100 per cent transplantable.

Experiments for ensuring the absence of whole cells. In experiments designed

TABLE 2
FREQUENCY OF LEUKEMIA AFTER THE INJECTION OF G4 FILTRATES FURTHER
TREATED TO ASSURE THEIR BEING CELL-FREE

Preparation of the filtrates	Tumor	No. of mice after 6 mo.	No. of leukemias	Percentage of leukemias	Average latent period (days)
G4 filtrates, once filtered	Sa I	68	39	57	203
G4 filtrates, twice filtered		104	61	58	236
G4 filtrates, once filtered	Ehrlich Ca	90	34	38	243
G4 filtrates, twice filtered		12	8	~66	233
Membrane filtrates (0.6 μ diameter)	Sa I	11	6	~55	284
Supernatant after a twofold G4 filtration (centrifuged at 1360 \times g)	Sa I + SOV 16	29	20	~69	Sa I = 264 SOV 16 = 130
Supernatant after centrifugation at 20,000 \times g	Sa I	40	24	~60	315
Sediment after centrifugation at 20,000 \times g	Sa I	28	16	57	213

to show cell-free tumor transfer, it is of the utmost importance to demonstrate that the tumor extracts employed are free of cells. To this end the following procedures were applied to the G4 filtrates described above (TABLE 2).^{19, 21, 27-29} First, it was shown that the effectiveness of a twice-filtered tumor extract (G4 filter) was not diminished when compared with the once-filtered extract. Also G4 filtrates were very effective even after centrifugation at $1790 \times g$ and filtration through membranes impermeable to bacteria (diameter of the pores, 0.6μ). The same was true for the supernatants of the G4 filtrates after centrifugation at $1360 \times g$ and $20,000 \times g$. In some rare instances leukemias could also be produced with the supernatant of filtrates after ultracentrifugation at approximately $120,000 \times g$. It appears that these experiments definitely excluded the participation of whole cells. It is of interest however, that the sediments obtained in the high-speed centrifugation of the G4 filtrates also had strong leukemogenic activity. This indicates that the effective agent was partially sedimentable at speeds of $20,000 \times g$ and above, and thus may have particulate character.

Dependence of Leukemia Incidence on Certain Biological Conditions of the Experimental Animals

First, we were interested in determining whether there was a causative connection between the efficacy of the cell-free filtrates and the *genetic constitution* of the experimental animals.^{21, 31} G4 filtrates (filtered twice) were injected into newborn mice of the strains Agnes-Bluhm, sg, and db. The incidence of spontaneous leukemia in all three strains was below 1 per cent. The activity of the filtrates in the three strains was as follows: Agnes-Bluhm, 48 per cent; db, 41 per cent; and sg, 70 per cent—a total of 92 leukemias. No significant connection could be established between the sex of the animals and the incidence of leukemia (55 per cent of leukemias in the female and 45 per cent in the male).¹⁴ In addition, regarding the role of age upon the activity of the filtrate, no significant difference was noted in the susceptibility to the cell-free filtrates of tumor sarcoma I from birth to the 11th day.^{19, 31} Adult animals (age 3 to 4 months) were rather susceptible to sarcoma-I filtrates (22 leukemias in 76 surviving animals—29 per cent).^{31, 32} The leukemias here were also chloroleukemias, with an average latent period of 209 days.

Finally, we investigated whether whole-body irradiation with X rays influenced the effectiveness of the filtrate.³³ Adult mice were injected with a suspension of the sediment obtained from G4 filtrates of the weakly active tumor sarcoma 37 and were exposed simultaneously to a whole-body irradiation of 840 r. Of 23 surviving animals, 7 developed leukemias, of which 6 were characterized as chloroleukemias. In controls of approximately the same weight that received only X-ray treatment or were injected only with tumor material, no leukemias were observed. Therefore, the whole-body irradiation with X rays enhanced the leukemogenic effectiveness of the cell-free tumor material.

Carcenogenic hydrocarbons appear to have a similar effect.³⁴ In the experiments in which sarcoma 37 filtrates were applied simultaneously with

carcinogenic hydrocarbons (9,10-dimethyl-1,2-benzanthracene, benzpyrene, or methylcholanthrene) 25 per cent leukemias were observed (12 leukemias in 48 animals) as compared with 3 per cent and 7 per cent, respectively, in the animals treated either with carcinogens only or only with the filtrates. These experiments (combination treatment with X rays and carcinogenic substances) are of a preliminary nature.

Studies Regarding the Mode of Action of Cell-Free Tumor Filtrates

Two possibilities, in particular, must be taken into consideration: (1) a specific leukemogenic agent; and (2) a nonspecific injury to the animals by certain components of the filtrate, producing leukemia indirectly.

Indeed, it was necessary to take into consideration the latter possibility, since our standard dose (0.1 ml. G4 filtrate) contained about 1 mg. of protein, which is a rather large amount of material for a newborn mouse. We investigated, therefore, whether considerably smaller doses of the filtrate, very unlikely to produce a nonspecific damage, would still show leukemogenic activity. One hundredth of our standard dose (filtrate dilution 1:1000) still showed activity (8 leukemias in 31 surviving animals—26 per cent).^{25, 27}

If one assumes a nonspecific activity of the filtrates, one could also expect that not only filtrates of tumor tissues but also extracts of homologous and heterologous normal tissues, prepared in a similar manner, should be active. In these experiments with filtrates from a variety of normal tissues,^{19, 23, 35} the dosage was generally larger than that of active filtrates prepared from mouse tumors.

In 256 surviving animals treated with filtrates prepared from embryonic tissue of the mouse, the rat, or the chicken, and with heterologous sera, 14 leukemias appeared (5.5 per cent). Thus, the leukemia incidence was at least 10 times lower in these controls than that observed in experiments with mouse tumors. Also, no chloroleukemia was ever seen, and the latent period was 2 to 3 times longer than that in the leukemias produced by mouse-tumor filtrates. It should be noted, however, that the injection with filtrates prepared from normal tissue did increase to a certain extent the incidence of leukemia when compared with the incidence in nontreated controls of the same source, in which approximately 1 per cent spontaneous leukemia was found. Finally, control experiments were conducted by treating mice with filtrates obtained from heterologous tumors (Walker carcinoma and Jensen sarcoma of the rat).^{19, 35} Negative results were obtained in these experiments also (5 leukemias in 167 animals—3 per cent) and, at the same time, they indicated the presence of a specific leukemogenic agent in the mouse tumors employed. Moreover, these experiments with rat tumors also suggested the specificity of action of cell-free tumor filtrates. In the same manner, experiments must be evaluated in which filtrates of the tumor sarcoma I and SOV 16, which were very active in the mouse, were injected in high doses into newborn rats.³⁶ In 149 animals, most of which survived longer than a year, neither leukemias nor other tumors that could be related to an action of the filtrate were observed.

*Some Physical, Biochemical, and Biological Characterizations of the
Leukemogenic Agents*

The following experiments, with a few exceptions, are of a preliminary nature. In most instances tumor sarcoma I was used.

Heating the filtrate for half an hour at 65° C. destroyed the leukemogenic activity completely. Thus, the active agent was extremely sensitive to high temperatures (no leukemias in 33 surviving animals after an observation period of 1½ years).^{19, 29} On the other hand, the agent appeared to be rather resistant to low temperatures (−10° to −16° C.).³⁴ Frozen G4 filtrates, as well as filtrates prepared from frozen tumor material, produced leukemias (31 leukemias in 220 animals, or 14 per cent; 17 of the leukemias were chloroleukemias). In these experiments tumor sarcoma I, Ehrlich carcinoma, and sarcoma 37 were employed; the maximum period of freezing was 76 days.

The active agent was mildly sensitive to the irradiation of a mercury lamp (1 hour of irradiation decreased the tumor incidence from 66 to 16 per cent).³⁷

The nucleoprotein precipitate of the filtrates showed considerable leukemogenic action (5 leukemias in 13 surviving animals, or 38 per cent).^{25, 27} The precipitation was carried out at pH 4.5 to 4.8 (acetate buffer) followed by two reprecipitations at pH 9.5 (carbonate buffer). This experiment possibly can be considered as indicating the nucleoprotein nature of the agent. Ribonuclease (RNase) and deoxyribonuclease (DNase) isolated from tumor tissues and injected into newborn mice so far have shown no effect in a maximum period of observation of 10 months (1 leukemia in 256 surviving animals).³⁴ Our experiments in which crystallized RNase and DNase were added to the filtrate, with subsequent inoculation of the mixture under conditions favorable to these enzymes, likewise indicated that the free nucleic acids were inactive.³⁷ In the series where RNase was added, 38 per cent leukemias were observed (20 leukemias in 53 animals), as compared with 53 per cent (24 leukemias in 45 animals) in the control series without enzyme addition. In the experiments with DNase the corresponding values were 20 and 25 per cent. This very slight influence of the two enzymes employed upon the leukemogenic effect of the filtrate is in contradiction to the concept that the agent is composed of free nucleic acids.

Extensive investigations were conducted to show whether the leukemogenic agent could be propagated outside of the mouse organism. Employed were: (1) the chorioallantois of the incubated chicken egg^{25, 27} and (2) homologous tissue cultures (Maitland- and Roller-tube cultures).³⁵ The conditions and the tissues were varied in many ways. Cultures of embryonic whole mouse tissue, the thymus gland, and the bone marrow were injected with cell-free filtrates of tumor sarcoma I and SOV 16 and were subsequently incubated. G4 filtrates of the homogenized cultures and the chorioallantois of the chicken, respectively, were obtained after one or more passages injected into mice. Thus far, these experiments have been negative in regard to the production of leukemias (more than 500 surviving animals). It was quite

TABLE 3
INACTIVATION OF THE LEUKEMOGENIC FACTOR FROM A G4 FILTRATE OF THE TUMOR SA I
BY RABBIT ANTISERUM

	No. of surviving animals	No. of leukemias	Per cent leukemias
Sa I filtrate + rabbit antiserum	254	22	9
Sa I filtrate + rabbit normal serum	185	99	53
Sa I filtrate + Ringer's solution	190	88	46

remarkable, however, that these homogenates of the homologous embryonic tissues, particularly after many passages, were very toxic in spite of their bacterial sterility. The death rate of the injected newborn animals within the first two weeks was nearly 100 per cent. Such high toxicity was observed at times also in freshly prepared and sterile tumor filtrates, particularly in certain inoculation passages.¹⁹

In addition, immunological experiments for the characterization of the leukemogenic agent were conducted on a rather large scale.^{39, 40} Conditions similar to those in use by various scientists in experiments were thus employed with the Rous agent, the leukemia agent of the chicken, and with the Bittner factor. By immunization of rabbits with G4 filtrates or, even better, with sediments obtained by high-speed centrifugation of the filtrate, antisera could be obtained that practically canceled the leukemogenic effect of freshly prepared sarcoma I filtrates. The antiserum was mixed with the freshly prepared G4 filtrate immediately before injection into newborn mice. In the controls, either normal rabbit serum or Ringer solution was added to the filtrate in place of the antiserum. The addition of complement or short incubation of the mixture of antiserum, plus filtrate before injection, showed no significant increase of inactivation. On the other hand, the ratio of antiserum:filtrate was important. An increase of antiserum caused a corresponding increase of inactivation. The evaluation of all our neutralization experiments with antiserum (a total of 629 animals, including controls) showed that in the control runs (normal serum or Ringer solution) the leukemia incidence was five times as high as in the runs with the specific immune serum (TABLE 3). In the experiments with relatively larger amounts of antiserum (antiserum:filtrate 1:1, or 3:2), the leukemogenic activity was reduced to one twelfth of the control value (7 leukemias in 155 animals 5 per cent as compared with 72 leukemias in 119 animals 60 per cent). A rabbit immune serum against normal mouse tissue (lymph glands) inactivated sarcoma I filtrates only slightly (24 per cent of the leukemias with rabbit immune serum against lymphatic tissue as compared with 46 per cent of the leukemias with rabbit normal serum as control).

Furthermore, we attempted to prepare a homologous antiserum by

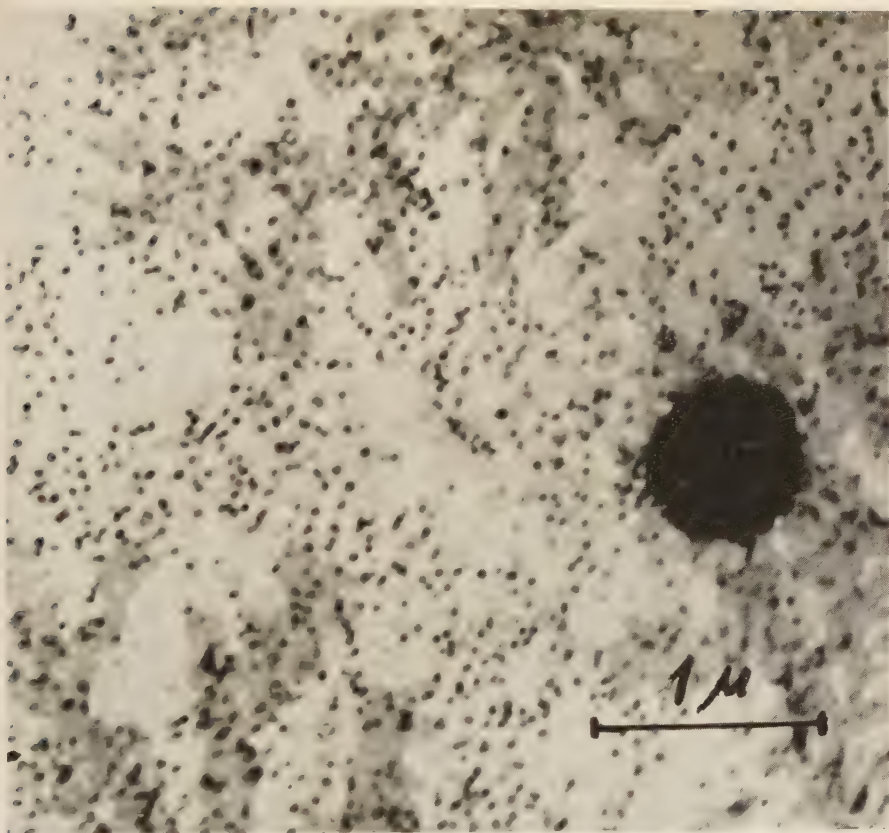


FIGURE 4. Electron micrograph of viruslike granular elements of the tumor Sa I—40 to 60 $m\mu$. $\times 30,000$.

immunization of adult mice with sarcoma I filtrate attenuated by heat or formaldehyde. This experiment was conducted in view of a possible active immunization against the leukemia agent. The serum obtained from these mice was mixed with active sarcoma I filtrate and was injected into newborn mice. So far, in comparison with normal mouse serum, no inactivation has been observed. The serum of immunized mice was also negative in the complement-fixation experiment with sarcoma-I filtrates, while heterologous antisera (rabbits, guinea pigs) up to a very high titer (1:2000) gave a positive reaction with sarcoma-I filtrate or high-speed centrifugation sediment. These experiments appear to demonstrate that either only very few or no homologous antibodies against the leukemogenic agent are formed.

I wish to report briefly a few electron microscopic investigations with tumor sarcoma I. Here, as before, no definite results were obtained. We found two viruslike elements that differed in form and size.^{19, 27, 29} The smaller particles were approximately circular and rather uniform (FIGURE 4). The diameter varied from 40 to 60 $m\mu$. The other somewhat larger element,

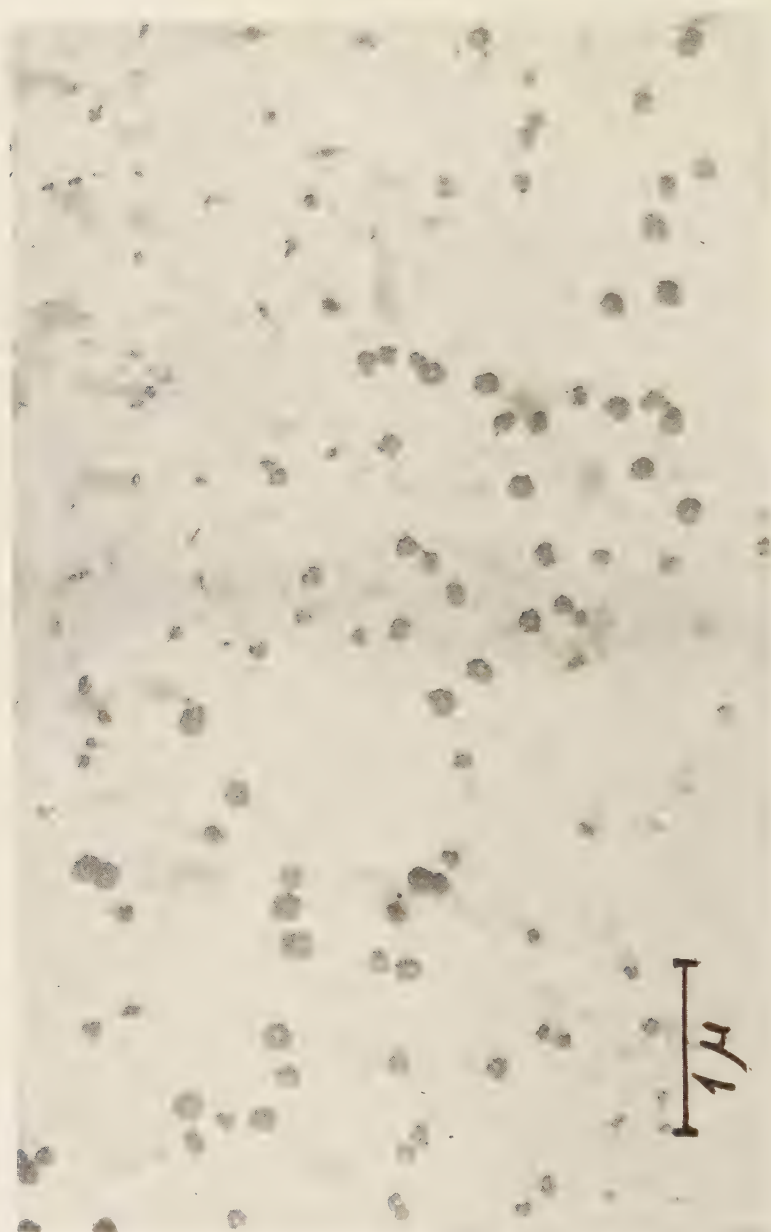


FIGURE 5. Electron micrograph of a smear of the sediment after centrifugation (50,000 rpm) of the Sa I-ascites serum (nonfixed). The corpuscular elements are 50 to 150 $m\mu$ in diameter, with a perforation in the center. $\times 20,000$.

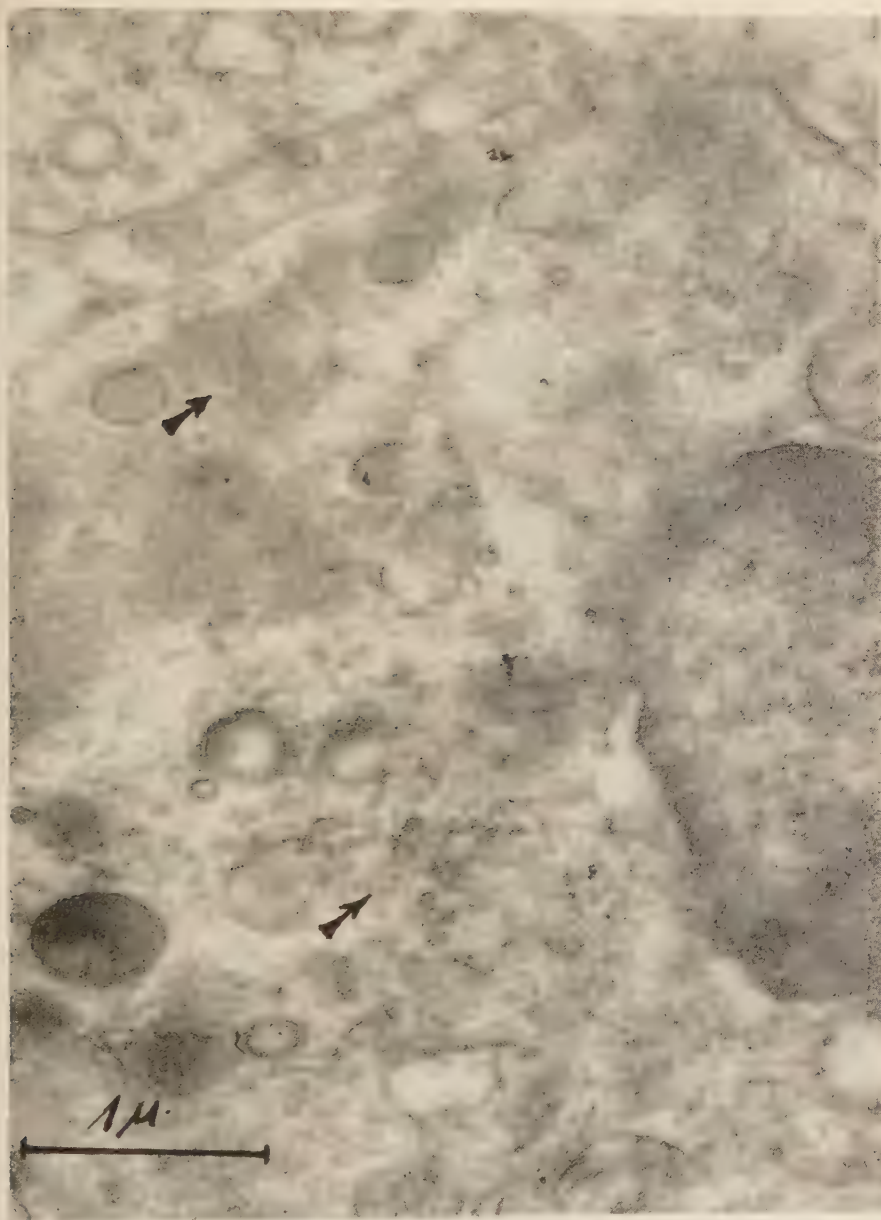


FIGURE 6. Section from a cell of the tumor SOV 16. The nucleus is on the right. In the cytoplasm (arrow) are some irregular mitochondria and a large number of small vesicles of 40 to 150 $m\mu$ in diameter that could be microsomes, endoplasmic reticulum, or partly viral elements corresponding with the elements of FIGURE 5.

particularly in the high-speed centrifugation sediments of the serous ascitic fluid of sarcoma I (FIGURE 5), has a diameter of approximately 50 to 150 $m\mu$ and is characterized by a somewhat irregular contour and a central perforation. In the ultrathin slice of tumor SOV 16, small bubblelike structures of about equal size could be recognized (FIGURE 6).⁴¹ It must remain an open question whether these structures are viruses related to leukemia, or companion viruses, or normal cell constituents, such as microsomes. It should be noted, however, that the cell-free and twice-filtered ascitic fluid of tumor sarcoma I, in which these particles were found, had a very high leukemogenic effectiveness (22 leukemias in 39 surviving animals—56 per cent).^{25, 34}

Summary

These results are closely related to the fundamental results of Gross.^{11-15, 42-45} The essential difference exists in the fact that, primarily, Gross observed lymphatic leukemias, while we observed myeloid leukemias. Also, our active cell-free extracts were prepared from nonleukemic tissue, that is, from transplantable carcinomas and sarcomas. In many other respects, however, such as, mode of transfer, inactivation by heat, probable particulate nature, and electron microscopic observations, our findings are similar to those of Gross, and also those of Friend,⁴⁶ Stewart,^{47, 48} Parsons,^{49, 50} Schwartz *et al.*,⁵¹ and Lohmann and Schmidt^{52, 53} of our Institute. In regard to the viruslike nature of the leukemogenic agent, it is still a moot question whether we are dealing with a real exogenous virus or an endogenous viruslike agent, possibly derived from certain normal cell organelles (particles of mitochondria, microsomes). We have considered the latter possibility for some time.^{8-10, 54} The recent excellent experiments by Eckert *et al.*⁵⁵ strongly support such a hypothesis.

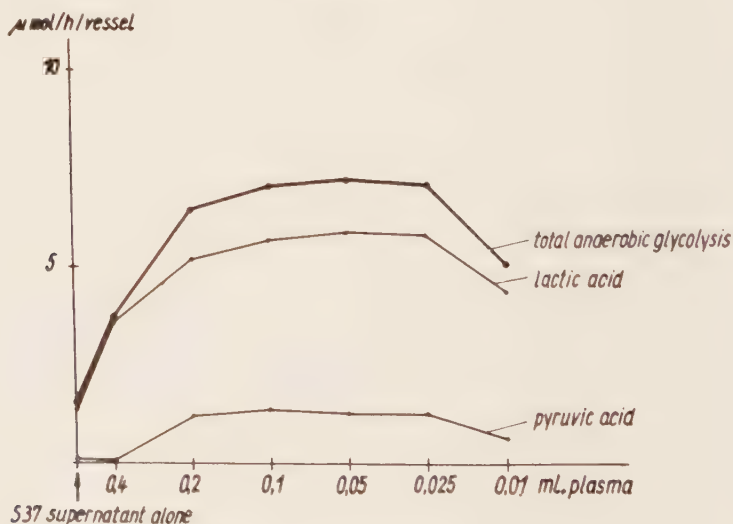


FIGURE 7. Plasma of chicken with myeloblastosis plus S 37 supernatant.

Finally, a few remarks may be permitted concerning a possible mode of action of oncogenic viruses upon the cell in the neoplastic process.⁵⁶⁻⁵⁹ These should be regarded as a working hypothesis.

We can show (FIGURES 7, 8, and 9) that the anaerobic glycolysis of the supernatant of a homogenate obtained by high-speed centrifugation of the mouse sarcoma 37 is intensified by addition of a suspension of myeloblastosis virus of the chicken.⁵⁹ The virus was kindly given to us by J. W. Beard, and has, according to Eckert *et al.*⁵⁵ and Mommaerts *et al.*,^{60, 61} a strong

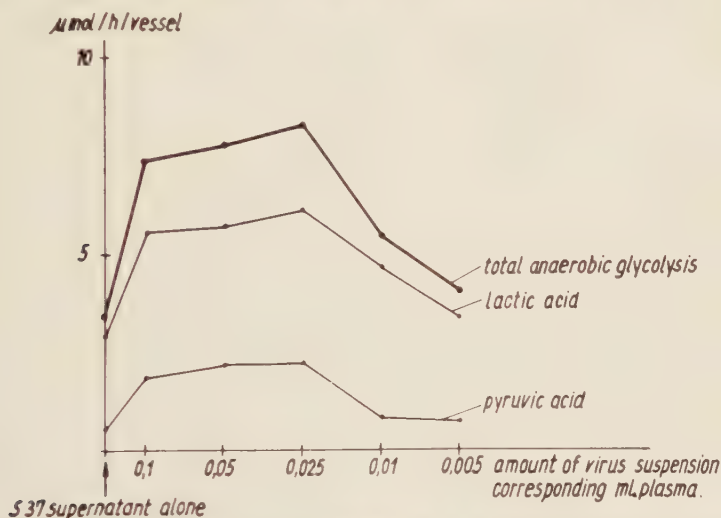


FIGURE 8. Ultrasediment suspension $120,000 \times g$ of cell free plasma of chicken with myeloblastosis plus high-speed S 37 supernatant.

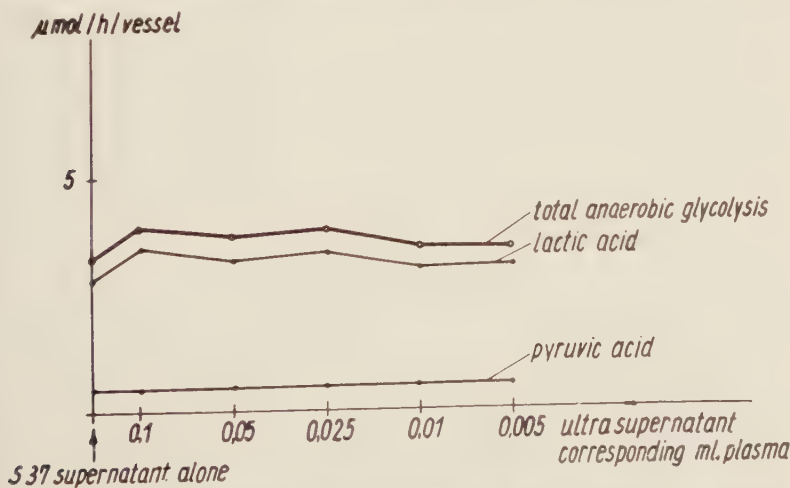


FIGURE 9. Plasma supernatant of chicken with myeloblastosis after ultracentrifugation plus high-speed supernatant of the tumor S 37.

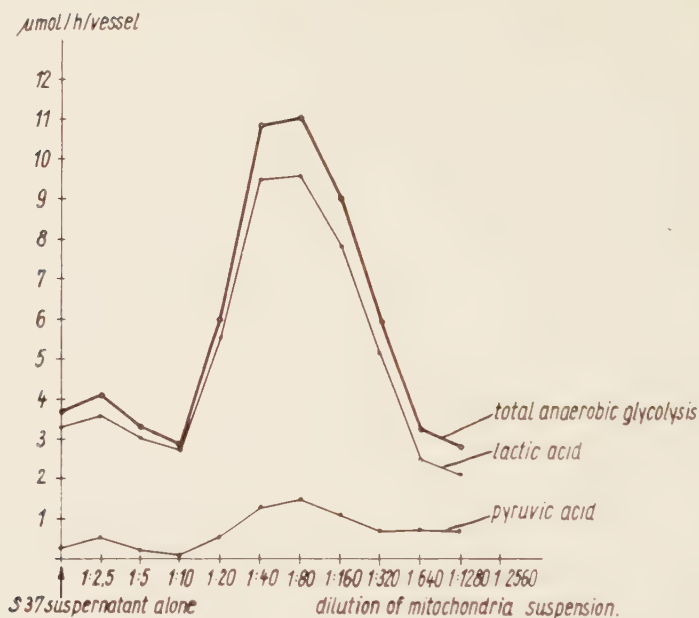


FIGURE 10. Jensen Sa mitochondria plus S 37 supernatant.

adenosinetriphosphatase (ATPase) activity. The same activity upon glycolysis is shown also by suspensions of isolated mitochondria,⁵⁸ particularly from malignant tumors (FIGURES 10 and 11), which also show a strong ATPase or apyrase activity. The anaerobic formation of lactic acid and pyruvic acid is increased more than 500 per cent by the addition of mitochondria in optimal concentrations. This stimulation of the glycolysis is dependent upon the concentration of these particles, whether myeloblastosis virus or mitochondria are involved. High concentrations have a lesser or even an inhibiting effect. A maximal increase of glycolysis is produced by a relatively low concentration of particles. We believe that this stimulating effect is a result of the ATPase activity.

We now propose that the principle common in carcinogenesis is the irreversible increase of an intracellular ATPase activity by which the glycolysis, and with it, the growth of cells, is stimulated.⁵⁶⁻⁵⁸ One could imagine that the ATPase activity is enhanced in the following two ways:

(1) By an irreversible structural damage of those cell organelles that have a latent ATPase activity (mitochondria, microsomes, and possibly certain parts of the cell nucleus), whereby their ATPase is activated in the same manner that has been observed in the swelling process of mitochondria *in vitro*. Such irreversible structural damage could be set off by chemical and physical carcinogens. Many of these, for example, the polycyclic hydrocarbons, seem to alter in particular the mitochondria and the microsomes.⁹ Such an effect upon the above-named structures of the cell, however, could also be brought about by a quantitative diminution of the virus infection.⁵⁸

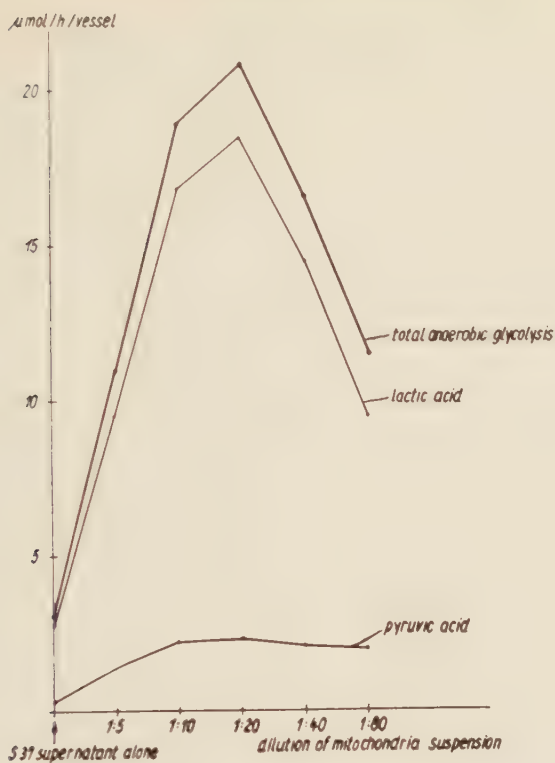


FIGURE 11. Ehrlich Ca mitochondria plus S 37 supernatant.

(2) By the intrusion into the cell of a virus that itself has ATPase activity. The latter can be assumed definitely in the myeloblastosis virus and other oncogenic viruses. Such an assumption would explain the bridging of the mode of action of chemical and physical carcinogens with that of certain viruslike agents.

I apologize for having allowed myself to leave the solid ground of facts with these speculations. One surrenders too easily to the temptation to try to explain carcinogenesis from a uniform point of view.

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SUBCELLULAR STRUCTURES OF POSSIBLE VIRAL ORIGIN IN SOME MAMMALIAN TUMORS*

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It is now a well-known fact that recent advances in electron microscopy have greatly contributed to our knowledge of the structure of normal cells and have laid foundations for studies of malignant cells on a submicroscopic level. In the natural course of events some of the first cells to be examined were those of tumors of known or suspected viral etiology.

Electron microscope studies have supplied a considerable amount of detail about cell components already known and have led to the discovery of new components that are now included in the structure of cells as normal cell constituents. It will suffice to enumerate these constituents briefly: mitochondria, ultramitochondria or ultrachondriom, ergastoplasm or endoplasmic reticulum, Golgi apparatus, small filaments, and small microsomes or Palade granules (for review, see Dmochowski, 1956a). Knowledge of these normal cell constituents has facilitated the study of cells and tissues infected by known viruses and has given, for the first time, a true picture of these subcellular or submicroscopic particles in their natural surroundings. It was therefore hardly surprising, but nevertheless extremely fascinating, to see that, in appearance, these subcellular particles were not as simple as we had been led to believe from previous electron microscope studies. Indeed, although subcellular in size, they have been found perhaps as complicated in their structure as the cells they infect. This characteristic, complicated structure has served quite a useful purpose in helping to differentiate these particles from known normal cell constituents. Unfortunately, we are far from understanding the significance and meaning of the structure of viral particles. This understanding may serve, in due course, as a meeting ground of biochemical and biological studies of subcellular particles, which leads to a point that can hardly receive too much emphasis: no matter how exciting electron microscope pictures of subcellular particles may look, their significance can be properly assessed only if combined with suitable biological, biochemical, and other studies. This point is particularly pertinent to studies on subcellular particles of viruslike appearance that may be found in neoplastic cells.

The amount of literature pertaining to the structure of viruses as seen in ultrathin sections of virus-infected tissues is constantly growing (Dmochowski, 1956a). It will suffice here to mention that the structure of known

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viruses, such as herpes simplex (Morgan *et al.*, 1954a), vaccinia and fowl pox viruses (Morgan *et al.*, 1954b), herpes B virus (Reissig and Melnick, 1955), and meningopneumonitis virus (Gaylord, 1954) resembles that of sub-cellular particles in mammalian tumors of known viral origin.

One of the first mammalian tumors of known viral etiology to undergo an extensive study by means of the ultrathin sectioning technique were those of mammary cancer in mice. The availability of purebred homozygous strains of mice with a known high or low incidence of breast cancer helped to provide not only ample material for such studies, but also suitable control material from strains of mice known to be virus-free and showing a low or comparatively low incidence of mammary tumors. Furthermore, the availability of suitable test mice, known to be virus-free but susceptible to the mammary tumor-inducing virus, gave convenient means for testing biologically the tumors, especially the control tumors, that had been examined in the electron microscope for the presence of the tumor-inducing virus.

For the past three years we have been engaged in an extensive study of mammary tumors from various strains of mice (Dmochowski *et al.*, 1953, 1954a, b, 1955; Dmochowski, 1954). A total of 56 mammary tumors, 23 from virus-containing and 33 from apparently virus-free strains of mice, was examined in approximately 2000 sections of these tumors. At the same time, the progeny of tumorous mice from virus-containing strains was observed for the incidence of mammary cancer, and tumors from apparently virus-free strains were tested by means of bioassays in (C57 \times RIIIb) F_1 hybrid mice and in suitable susceptible test mice of the C57 Black strain.

As the results of electron-microscope studies* have already been described, attention will be paid to some details pertinent to the present discussion, but not previously mentioned. The results of biological tests of control tumors, briefly reported on (Dmochowski, 1956a, b, c), will now be discussed in detail.

Characteristic viruslike particles were observed in 18 out of 23 tumors (78 per cent) of 3 different strains of virus-containing mice. They were also found in 11 of 33 breast tumors (33 per cent) of apparently virus-free strain mice from 3 different strains. To recapitulate, the particles found had an appearance similar to that seen in ordinary viruses. They showed an internal dense region and a well-defined outer membrane. They varied in size from 700 to 1450 A., and the internal dense core varied from 250 to 700 A. The size of the particles could vary in different sections of the same specimen of tissue or in sections of different specimens of the same mammary tumor. In addition, particles of a vesicularlike appearance and smaller in size, varying from 600 to 1000 A. in diameter, were observed. Structures that resembled inclusion bodies in virus-infected tissues were also found.

The appearance of some of the mammary tumor cells is shown in FIGURE 1. Part of the cytoplasm of two adjoining cells separated by cellular membrane may be seen, as well as part of the nuclei of both tumor cells. The ergastoplasm (Bernhard *et al.*, 1952; Weiss, 1953; Bernhard, Gautier, and Rouiller, 1954), or endoplasmic reticulum (Palade and Porter, 1952, 1954), appears

* An RCA EMU 3A electron microscope was used in the present study.

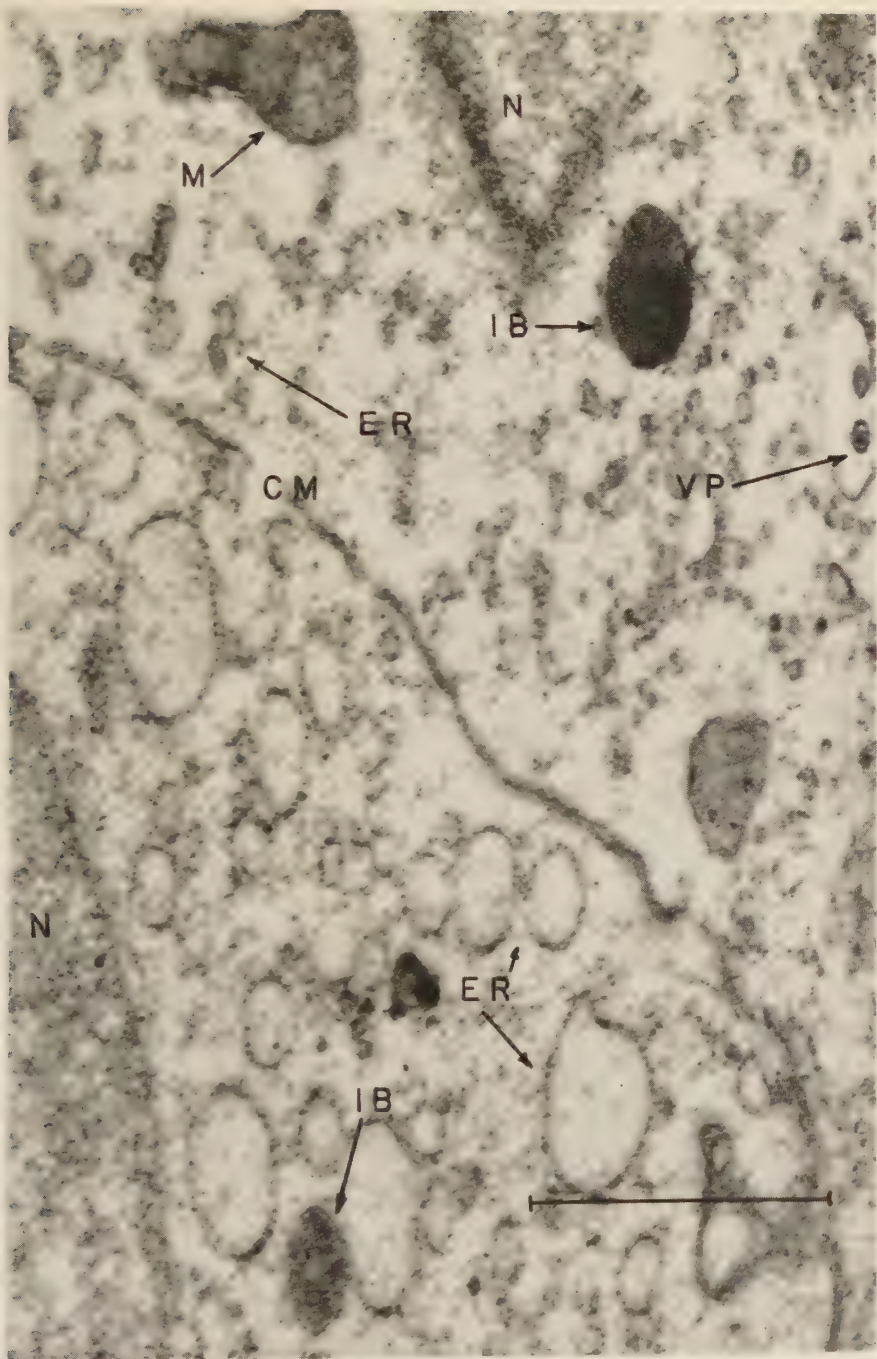


FIGURE 1. Mammary tumor of a C3H high-cancer strain mouse. N = nucleus; ER = ergastoplasm with Palade particles lined up along the walls of the vesicles; CM = cell membrane; M = mitochondrion; IB = inclusion bodies; VP = viruslike particle. $\times 38,000$.

to be considerably extended, forming vacuoles in the cytoplasm of one of the cells and, to a much smaller extent, in the cytoplasm of the other cell. Submicroscopic particulates, or Palade granules, or small microsomes (Palade, 1953a, 1955) are lined up along the walls of the vacuoles. The structure of the mitochondrion is not clearly defined, but in sections of other cells it could be seen with internal membranes, or cristae (Palade, 1952, 1953b), either discontinuous or continuous across the mitochondrial cavity, as described by Sjöstrand (1953, 1954), Sjöstrand and Rhodin (1953), and Rhodin (1954). The size and shape of the mitochondria was found to be variable. In FIGURE 1, two large-size osmiophilic bodies may be seen in the cytoplasm of the two cells, in one of which the formation of particles may be barely discerned. In the cytoplasm of one of the cells a viruslike particle with a dense center may be seen in what appears to be a vacuole. The number of viruslike particles varied considerably in different sections of the same tumor and in sections of different mammary tumors, from a few scattered throughout the cytoplasm, to a considerable number both in the cytoplasm and in the vacuoles of the cytoplasm, and outside the cytoplasm in the intercellular spaces or ducts.

A considerable number of particles with the characteristic structure may be seen in the vacuoles present in the cytoplasm of another cell shown in FIGURE 2. In addition, an aggregation of vesicular-type particles, smaller in size than the characteristic particles, is present in the cytoplasm partially surrounding what appears to be an inclusion body. Two characteristic viruslike particles may be seen nearby. The mitochondria that are present appear to have discontinuous membranes. A somewhat similar picture may be seen in FIGURE 3. A number of characteristic particles, surrounded by several cells, is present in the duct. In the cytoplasm of one of the cells an aggregation of vesicular-type particles and an inclusion body containing a characteristic viruslike particle are seen. In addition, some microvilli extending into the duct are visible; two of these are cut across and show a double membrane. The internal membrane is more dense than the outer membrane. On a number of occasions the macrovilli were seen extruding particles resembling characteristic particles into the lumen of the duct.

Microvilli extending into the duct may be seen in FIGURE 4. In the duct a number of spherical particles may be seen showing two such membranes as described in the previous figure. They may be cross sections either of microvilli or of the characteristic particles cut across at a certain level. A fully formed particle with a dense internal center is present among these structures. A similar picture, shown in FIGURE 5, deserves attention because of the presence in the duct of a characteristic structure with two dense centers surrounded by a single outer membrane. In addition, microvilli and characteristic particles, both in cross sections at different levels, may be seen. A considerable number of characteristic particles present in the intercellular space is shown in FIGURE 6. Measurement of the particle size in this section showed that the size of the whole particle varied from 830 to 1390 Å., with the size of the dense center varying from 280 to 690 Å. in diameter. The average size of the characteristic particles came to 1060 Å.,

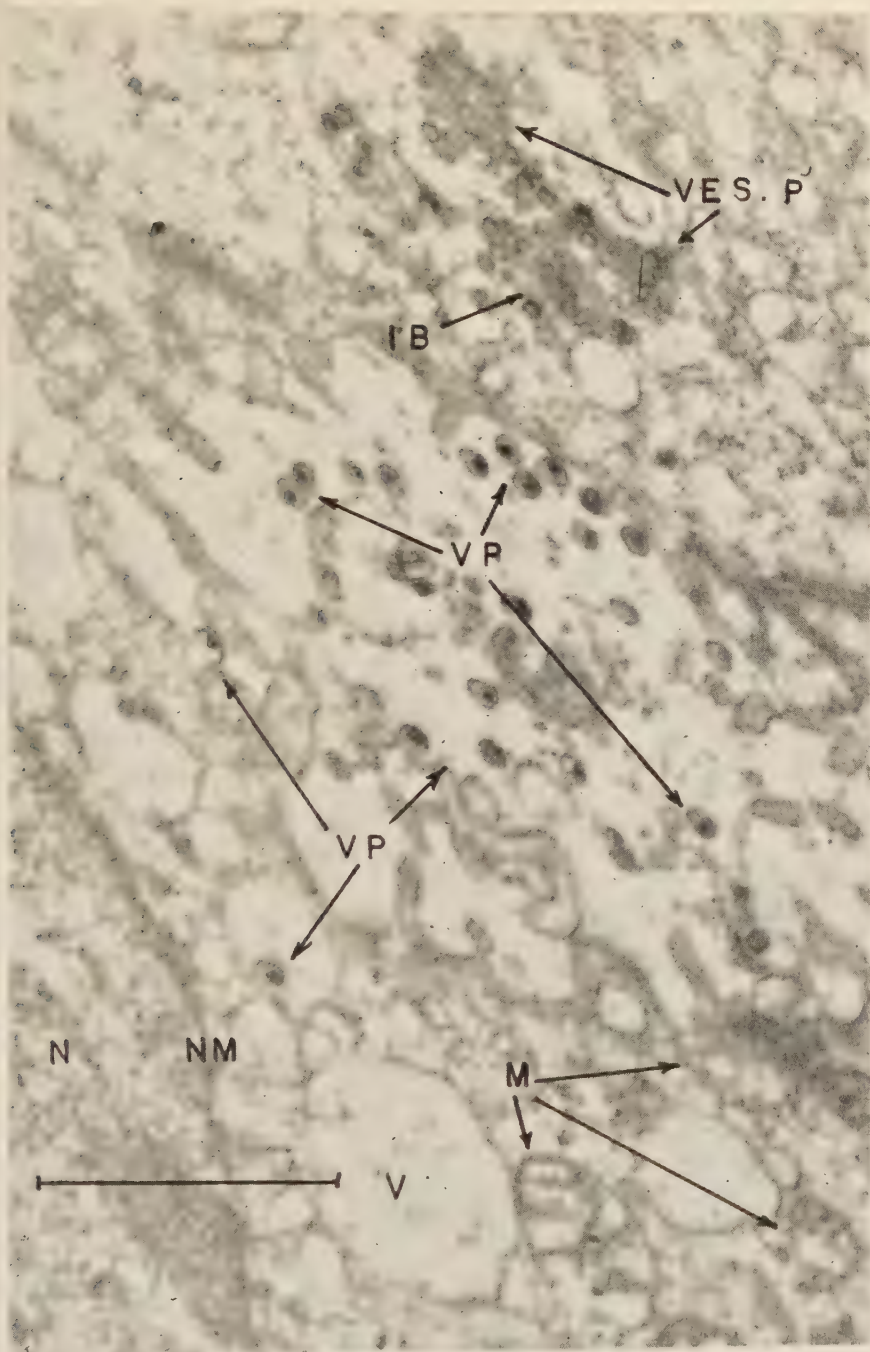


FIGURE 2. Mammary tumor of a C3Hf low cancer strain or apparently virus free strain of mice. N = nucleus; NM = nuclear membrane; V = vacuole; M = mitochondria; VP = viruslike particles; IB = inclusion body with outlines of particles inside the body; VESP = vesicular-type particles. $\times 38,000$.

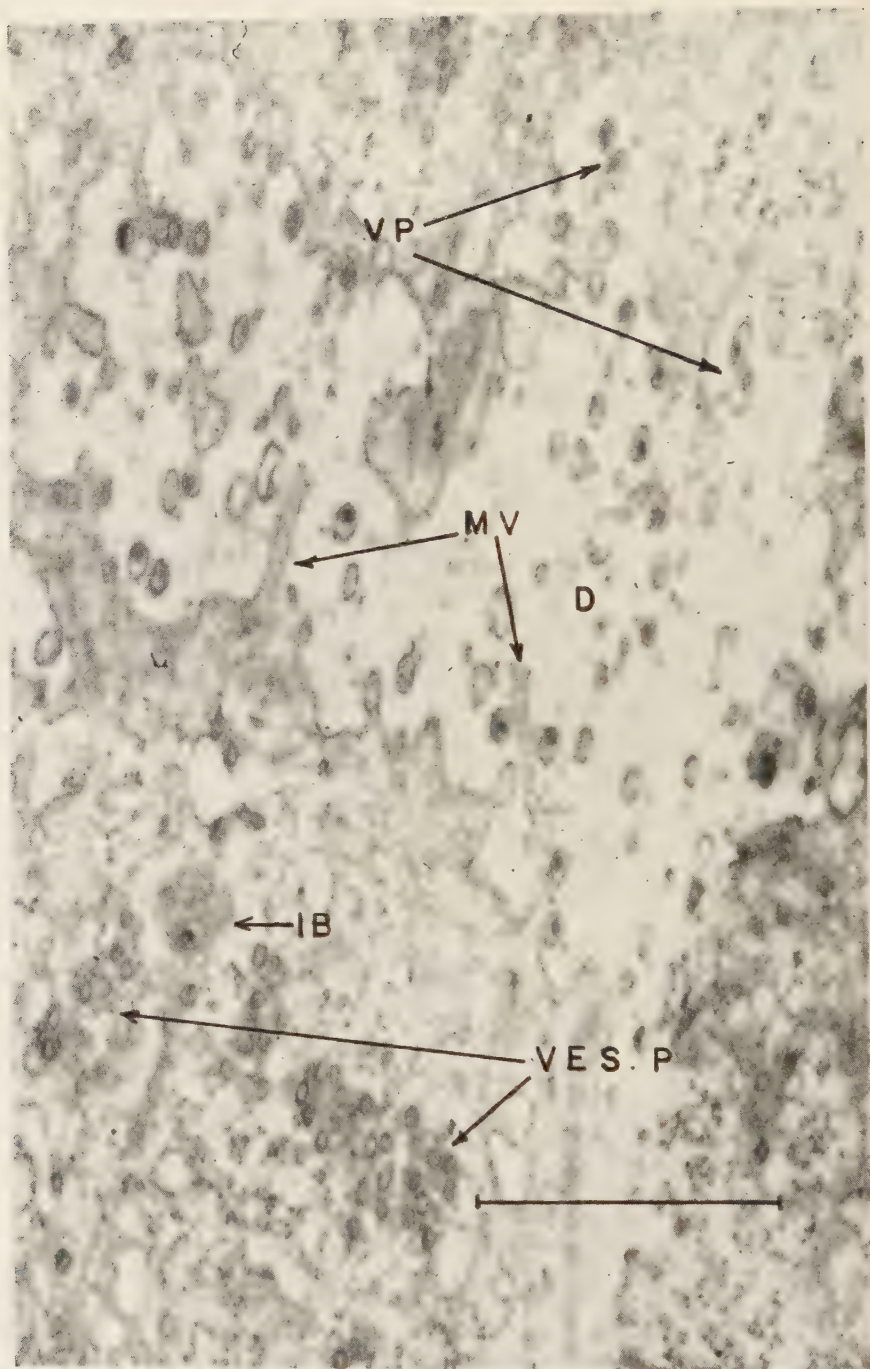


FIGURE 3. C3Hf breast tumor of an apparently virus-free strain of mice. VESP = vesicular-type particles; IB = inclusion body; MV = microvilli, some of which may be seen in the transverse sectioning plane; VP = viruslike particles in a duct (D). $\times 38,000$.

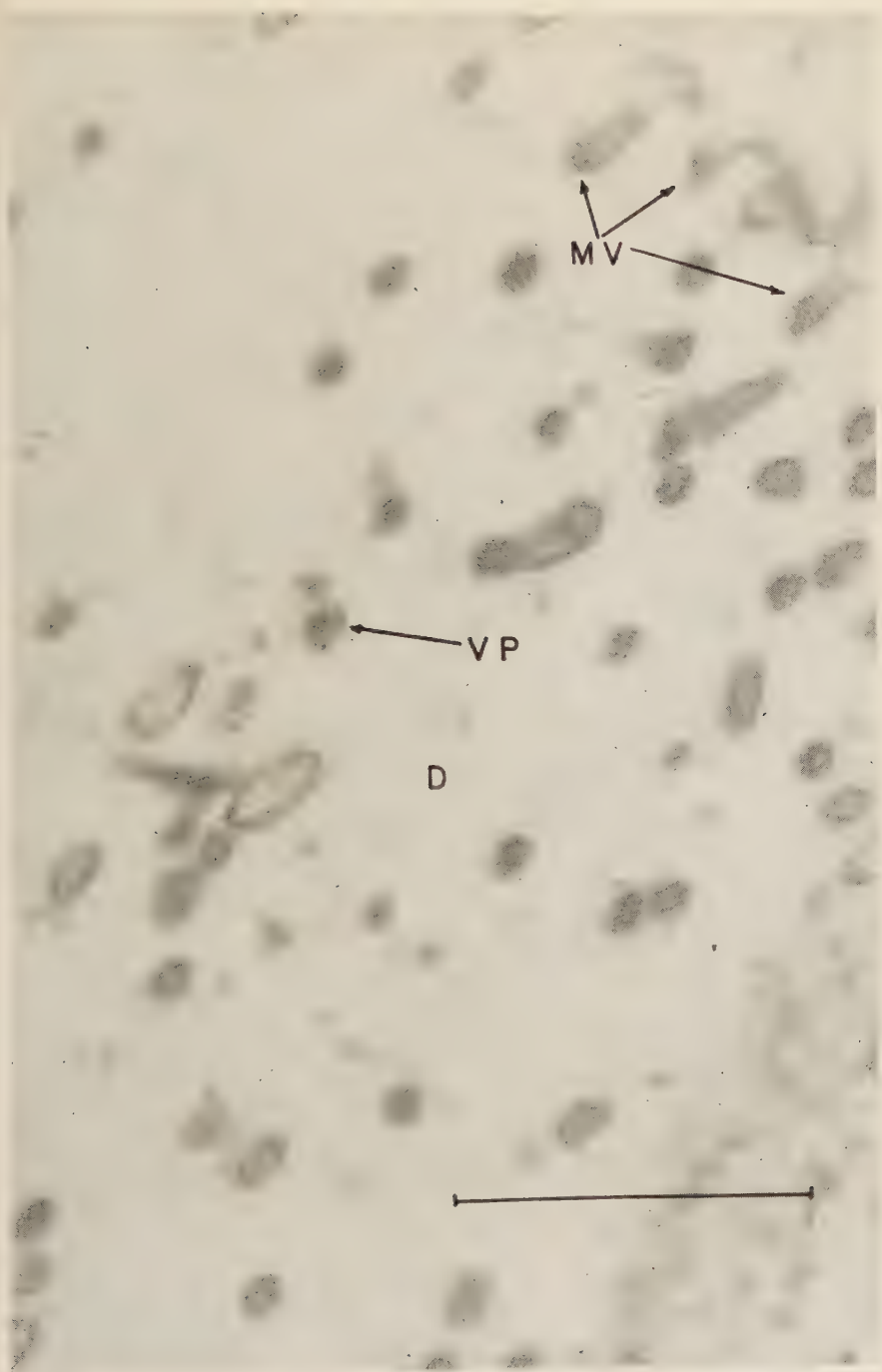


FIGURE 4. Part of a duct (D) in a lung metastasis of an RIII high cancer strain or virus strain tumor. MV = microvilli; VP = viruslike particle. Viruslike particles with double membranes and a transparent core are present in the duct. $\times 47,000$.

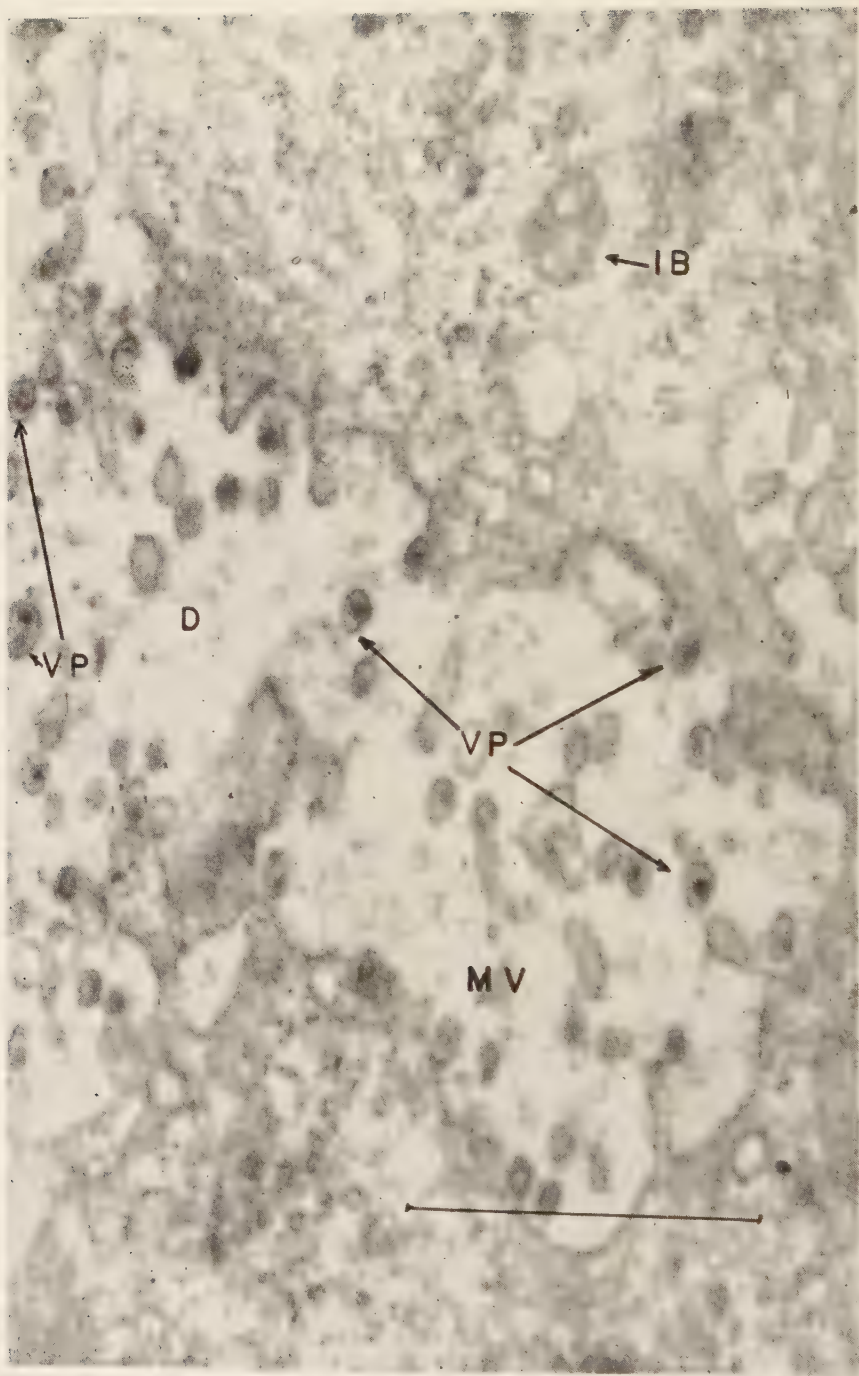


FIGURE 5. C3Hf strain mammary tumor of an apparently virus-free strain mouse. D = duct; VP = viruslike particles, with one showing two dense centers; MV = microvilli; IB = inclusion body. $\times 46,000$.

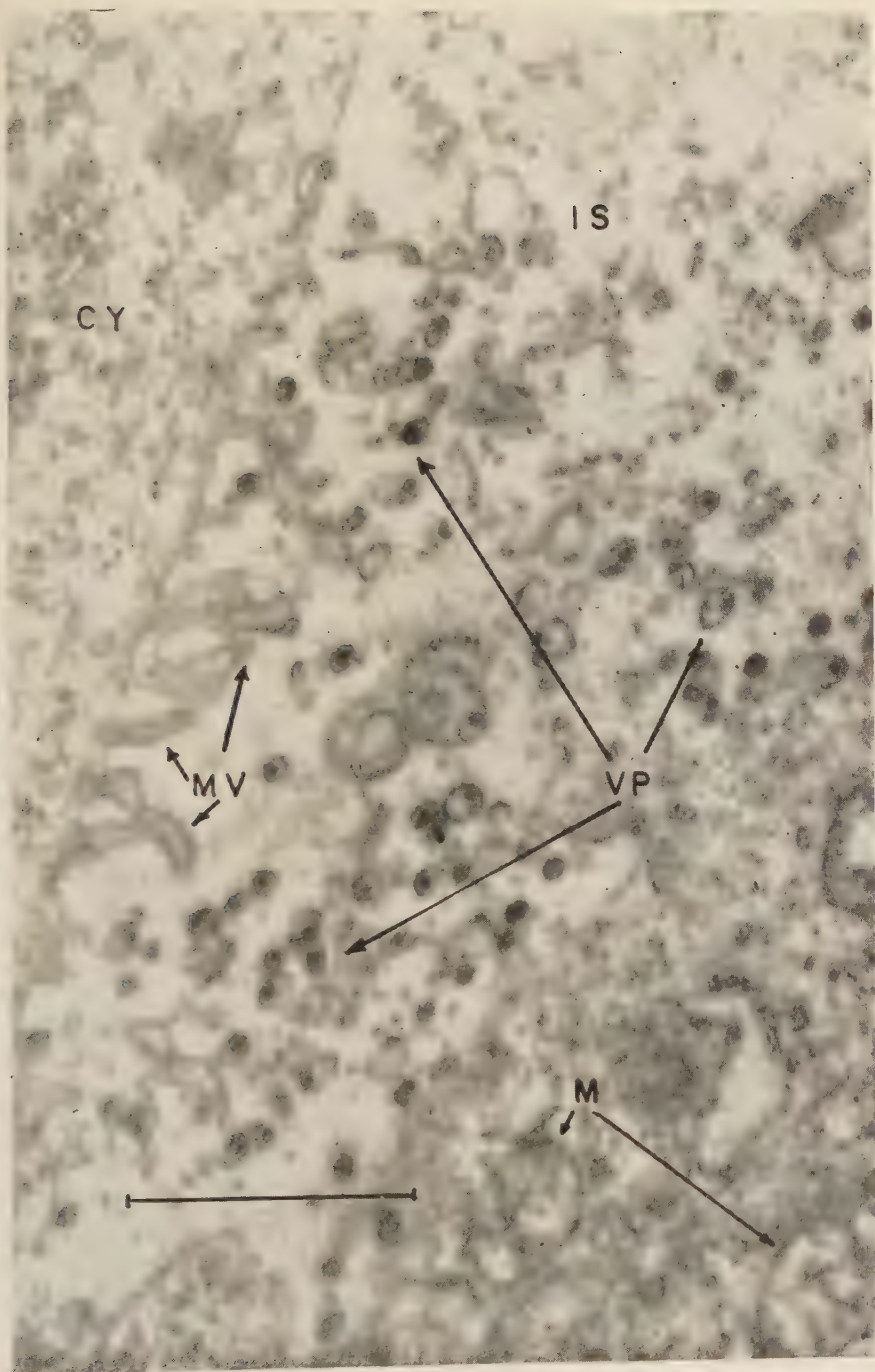


FIGURE 6. C3Hf strain tumor. VP = virus particles in an intercellular space (IS); MV = microvilli; CY = cytoplasm; M = mitochondria. $\times 36,000$.

and that of the center core to 500 Å. Projections of the cell membrane, described as microvilli, may also be seen in this section.

A well-preserved cytoplasm of several adjoining cells may be seen in FIGURE 7. Cellular membranes are clearly delineated, and the double nuclear membrane of the nucleus of one of the cells is also shown. The mitochondria present show internal membranes either continuous across the mitochondrial cavity or distorted, which may be due either to degenerative changes or to imperfect fixation. However, in view of the good preservation of other mitochondria lying in close proximity, the first possibility appears to be the more likely one. A considerable number of characteristic viruslike particles appears to surround what may be a degenerated mitochondrion. Similar changes in mitochondria of other neoplastic cells have been described by Howatson and Ham (1955). Submicroscopic particulates, described by Palade (1953a, 1955), are present in the cytoplasm.

A part of the cytoplasm of several adjoining mammary tumor cells, showing vacuolization and aggregation of vesicular particles around the vacuoles, is shown in FIGURE 8. In addition, what appear to be initial stages in the formation of inclusion bodies are present. Cellular membranes are also visible. FIGURE 9 shows a more advanced stage in the formation of inclusion bodies, with some characteristic viruslike particles. A part of the cytoplasm of two cells separated by cellular membranes may be seen. In the cytoplasm of one of the cells there is an aggregation of vesicular-type particles around the vacuoles; one of the vacuoles shows the presence of characteristic particles.

Almost all changes encountered may occasionally be seen in one section, as shown in FIGURE 10. Two neoplastic cells are seen with their cytoplasm separated by an intercellular space. Present in this space are characteristic viruslike particles, an inclusion body containing particles, and cross sections of several microvilli. Vesicular-type particles are also present. The nuclear membranes of both nuclei can be seen clearly. Measurement of the characteristic particles gave an average size of 1080 Å. (from 780 to 1320 Å.), with an average size for the dense center of 514 Å. (varying from 260 to 660 Å.). The variation in the size of the particles may well be due to differences in fixation.

The variation in the reaction of the cytoplasm with the formation of large vacuoles and the extension of ergastoplasm may be seen in FIGURE 11. In the cytoplasm of this neoplastic cell is an inclusion body and a large vacuole containing many characteristic particles.

The appearance of the vesicular-type particles at higher magnification is shown in FIGURES 12, 13, and 14. The particles measured in FIGURE 12 gave an average diameter of 800 Å., varying from 700 to 1000 Å. The nuclear membrane and the Palade granules are clearly visible in this figure. Some characteristic virus particles are also present. In FIGURE 13 an aggregation of vesicular-type particles may be seen lying either freely in the cytoplasm or around the inclusion bodies. Ergastoplasm and microvilli extending into a duct in another tumor cell separated by cellular membrane are also shown. FIGURE 14 shows an aggregation of vesicular-type particles in the

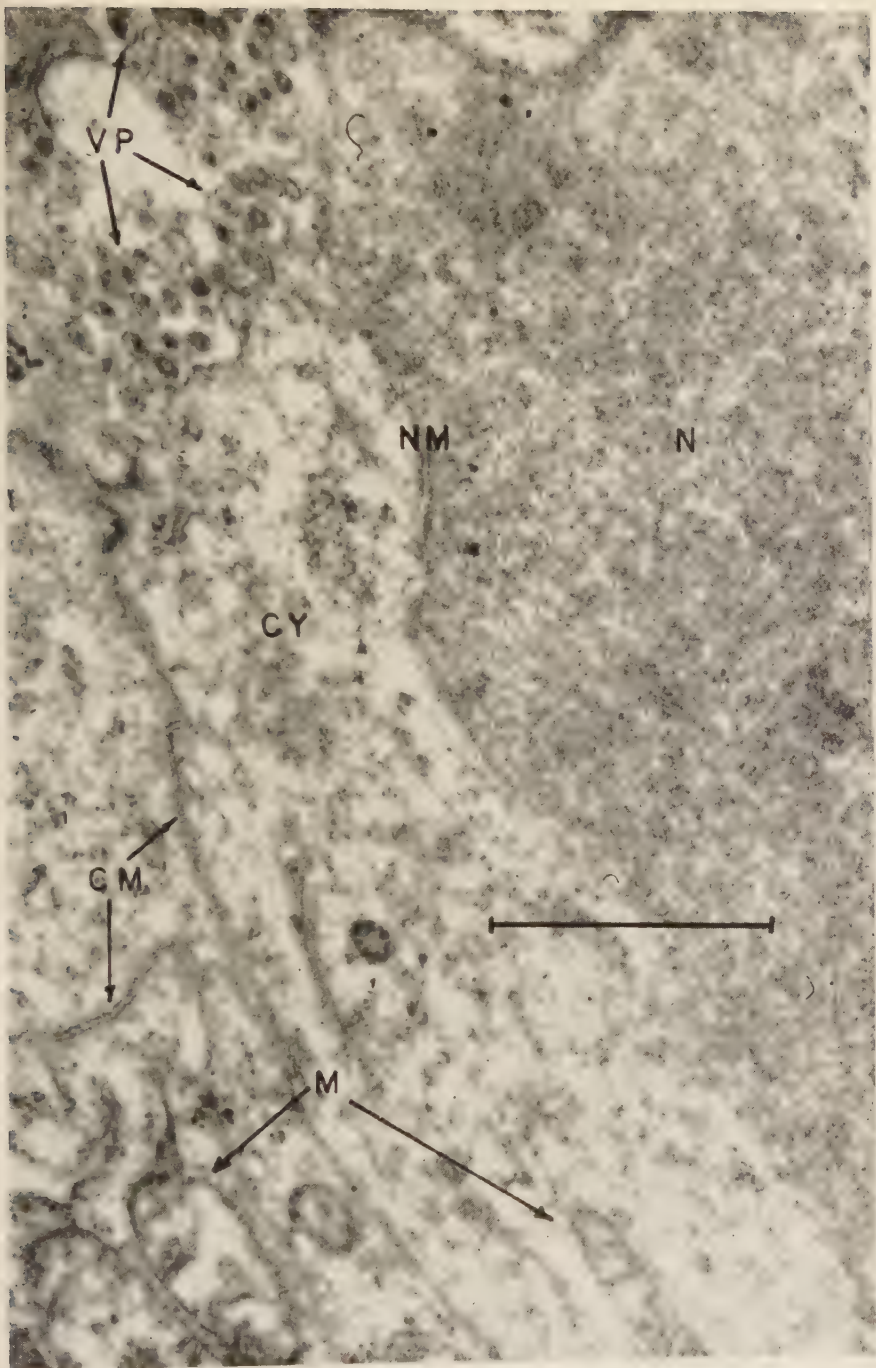


FIGURE 7. C3Hf strain mammary tumor. N = nucleus; NM = nuclear membrane; CY = cytoplasm; CM = cell membrane; M = mitochondria; VP = viruslike particles. $\times 36,000$.

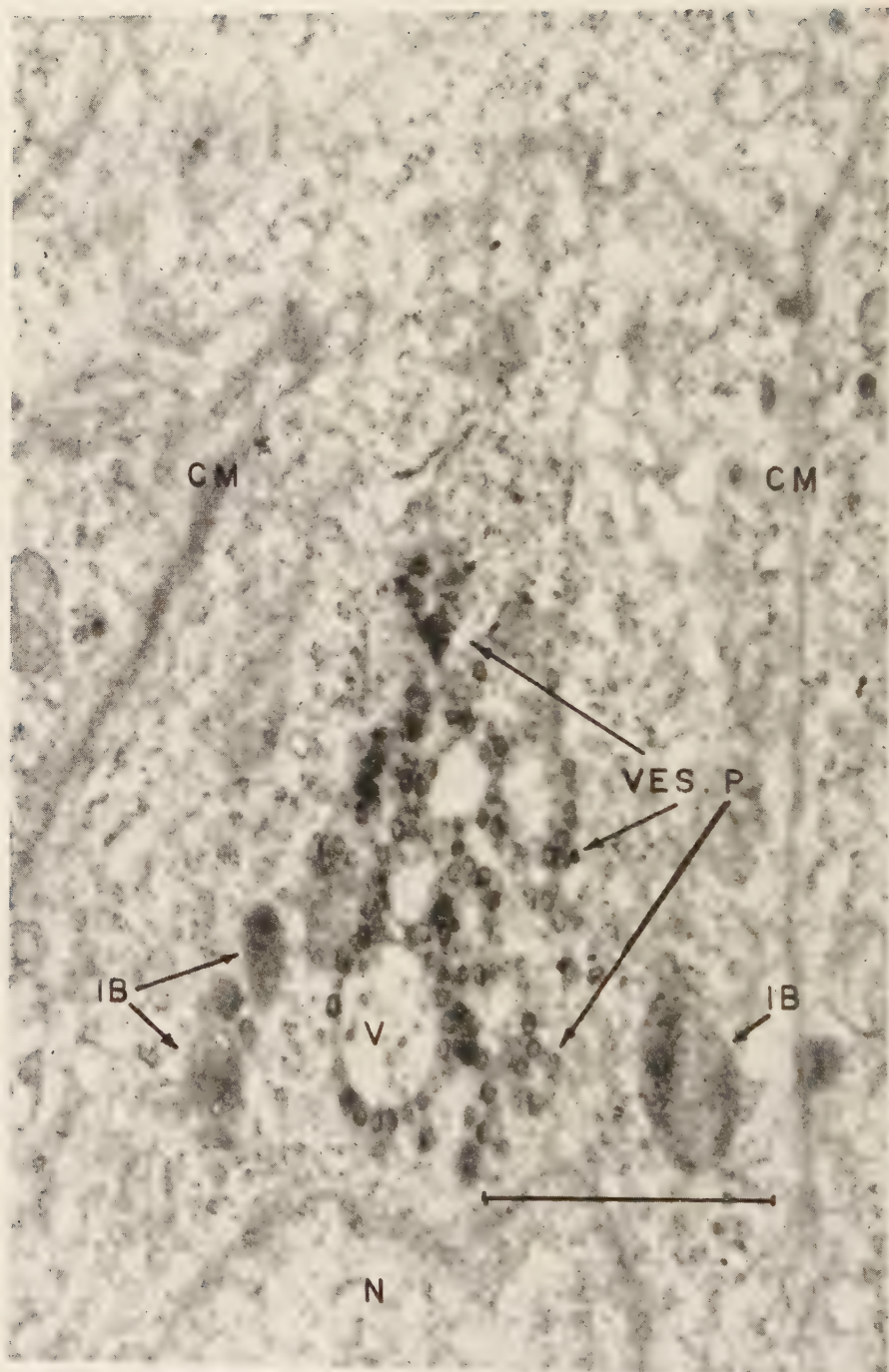


FIGURE 8. C3Hf strain breast tumor. N = nucleus; CM = cell membrane; IB = inclusion bodies; V = vacuoles; VESP = vesicular-type particles. $\times 36,000$.

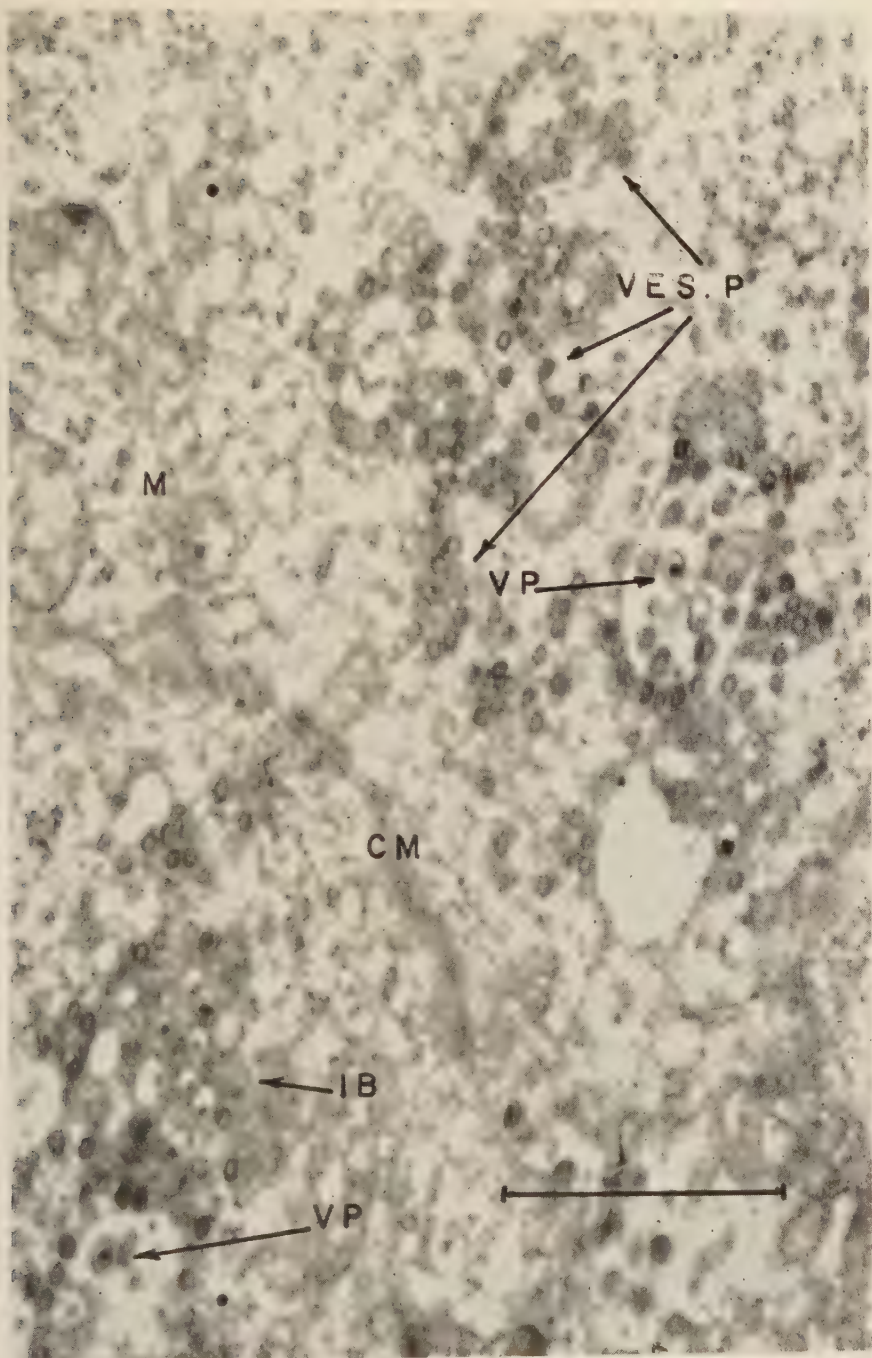


FIGURE 9. C3Hf strain breast tumor. IB = inclusion body; VP = viruslike particles; M = mitochondria; VESP = vesicular-type particles. $\times 36,000$.

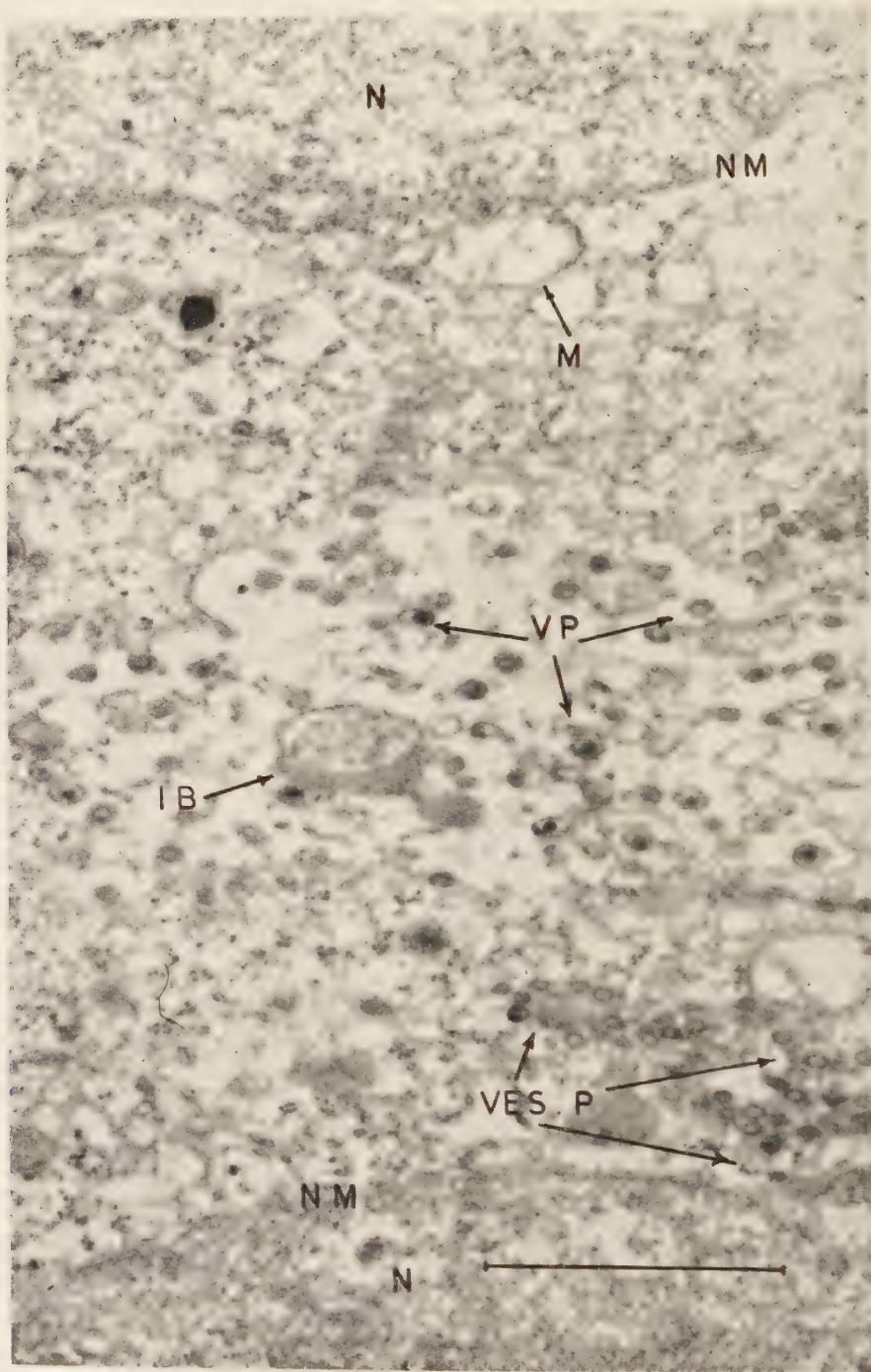


FIGURE 10. C3Hf strain breast tumor. N = nucleus; NM = nuclear membrane; VES. P = vesicular-type particles; IB = inclusion body; VP = viruslike particles; M = mitochondrion. $\times 38,000$.

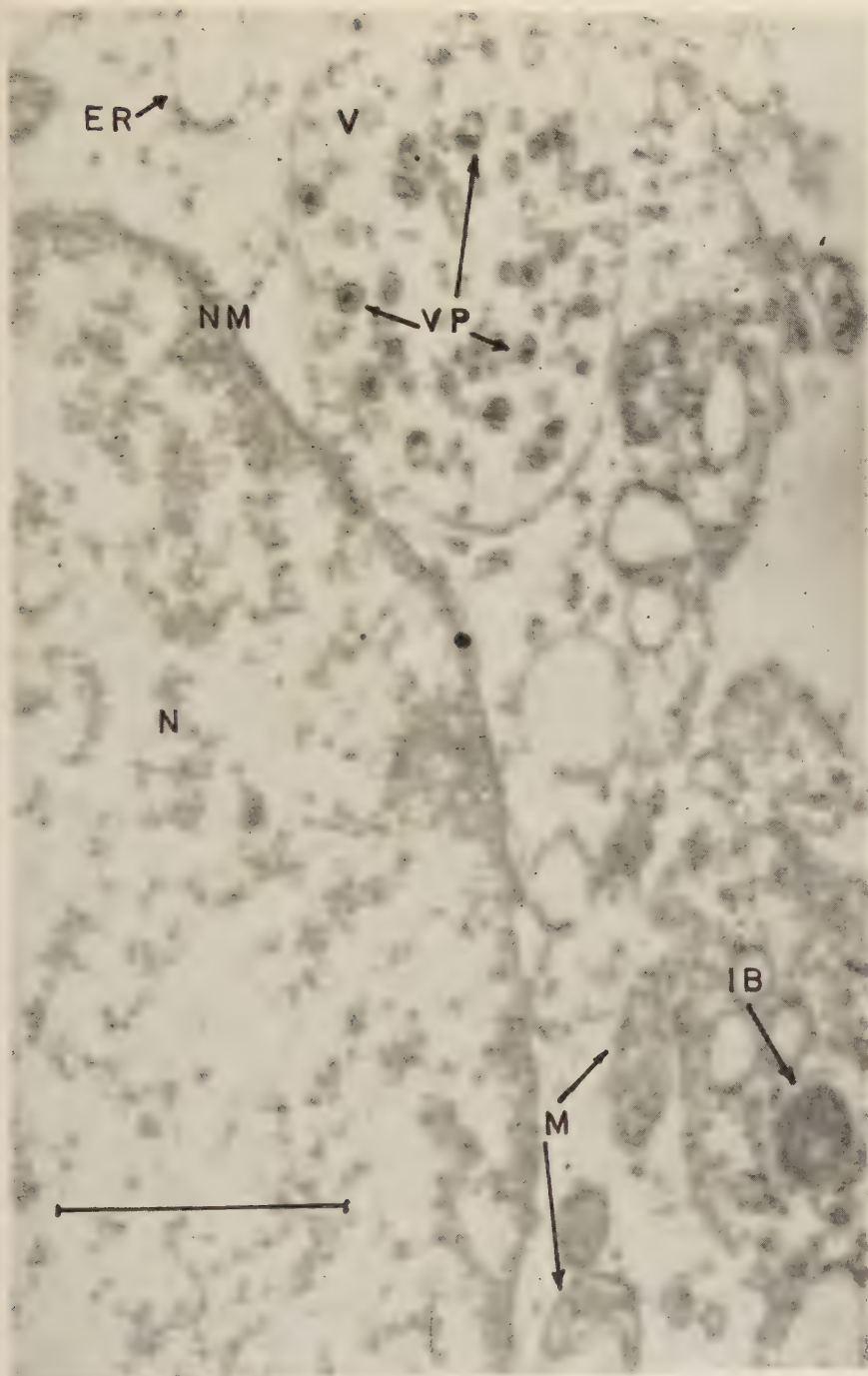


FIGURE 11. Mammary tumor of a C3H high-cancer or virus harboring strain mouse. N = nucleus; NM = nuclear membrane; M = mitochondria; IB = inclusion body; ER = ergastoplasm; V = vacuole; VP = viruslike particles. $\times 36,000$.

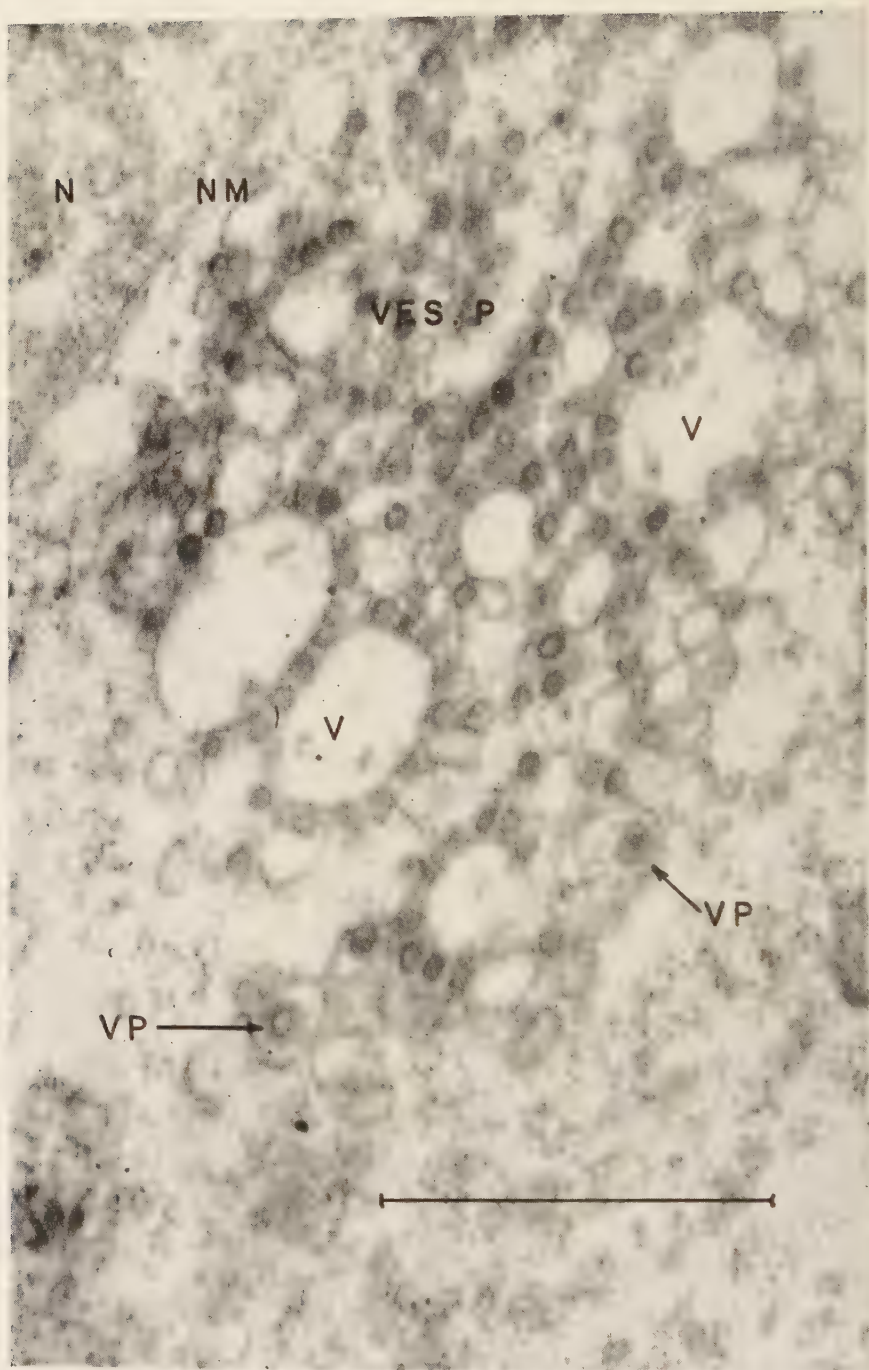


FIGURE 12. Mammary tumor of a C3Hf low-cancer or apparently virus-free strain mouse. N = nucleus; NM = nuclear membrane; VES.P = vesicular-type particles; VP = viruslike particles; V = vacuoles. $\times 50,000$.

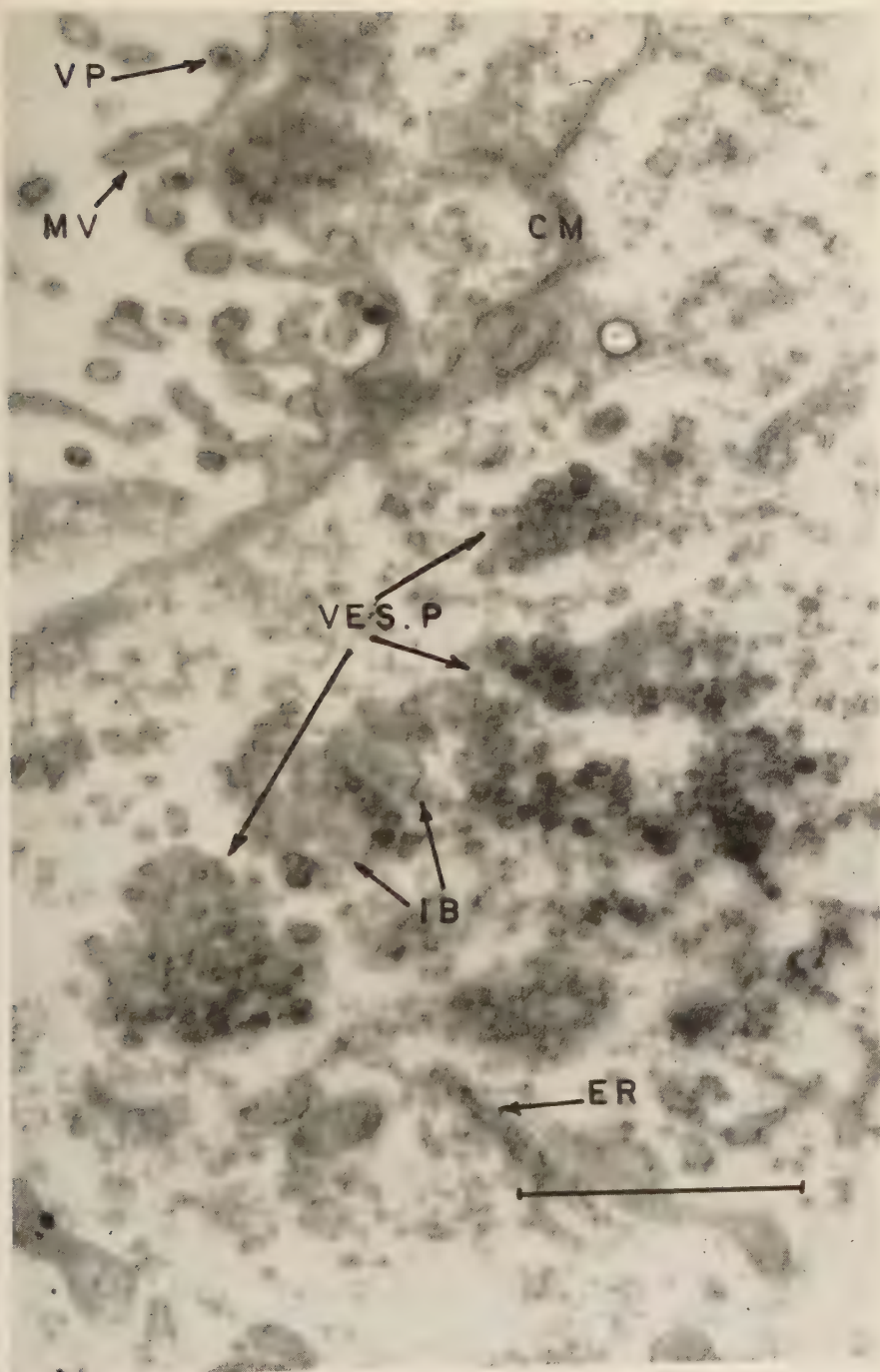


FIGURE 13. Mammary tumor of a C3H high-cancer or virus-carrying strain mouse. ER = ergastoplasm; IB = inclusion bodies; VESP = vesicular-type particles; MV = microvilli; VP = viruslike particles. $\times 36,000$.

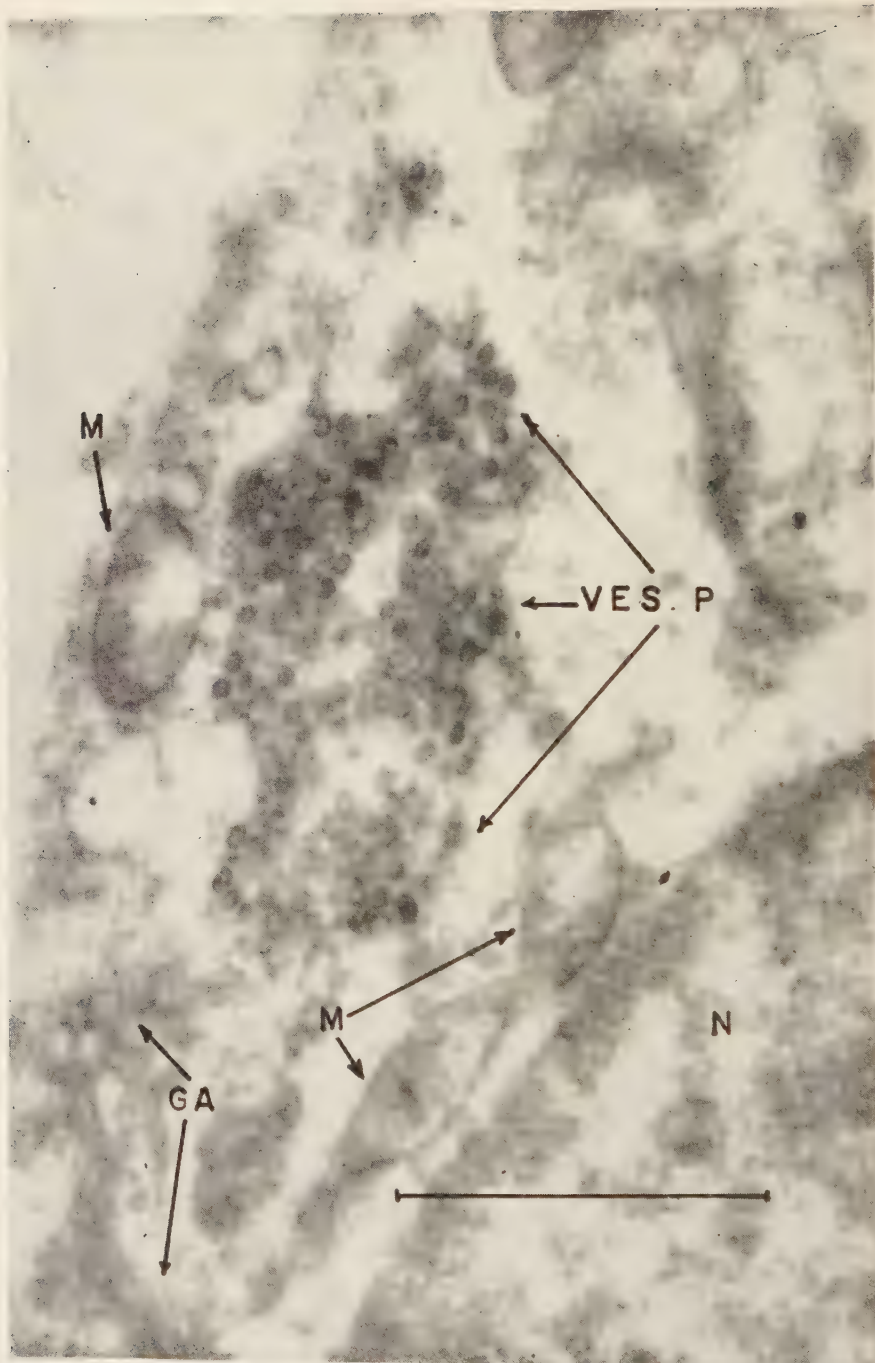


FIGURE 14. C3H high-cancer or virus-containing strain breast tumor. N = nucleus; GA = Golgi apparatus; M = mitochondria; VESP = vesicular-type particles. $\times 47,000$.

cytoplasm of a mammary tumor cell with mitochondria in various stages of degeneration. Also present in the cytoplasm of this cell is a system of parallel membranes, vacuoles, and granules that corresponds with the Golgi apparatus described by Dalton and Felix (1954), Sjöstrand and Hanzon (1954), and Haguenau and Bernhard (1955). The Golgi apparatus appeared to be enlarged in a number of cases, as observed by Haguenau and Bernhard (1955).

In spite of the changes observed in the mitochondria, the ergastoplasm, and occasionally in the Golgi apparatus, the tumor cells have not been found undergoing destruction. The virus particles differ from normal cell components of the tumor cells and from casein and lipid particles present in lactating mammary glands of high- and low-cancer strain mice. The latter particles do not show any internal structure. This excludes the possibility that the characteristic particles represent a stage in the secretion of milk (Dmochowski, 1956c).

Bernhard *et al.* (1955) have described similar viruslike particles and inclusion bodies in the cells of mammary tumors of virus-carrying mice. They also observed two types of particles similar to those described. However, control mammary tumors from virus-free strain mice were not studied at the same time. These authors concluded that the particles may be the mammary tumor-inducing virus and that their study does not provide a proof that the particles are indeed the virus responsible for mammary tumors in mice.

Recently, Bang, Vellisto and Libert (1956) described particles similar in appearance in sections of six mammary tumors from virus-carrying mice. In this study the authors have tentatively identified the viruslike particles as virus particles. In several instances, contrary to our experience and to that of Bernhard *et al.* (1955), they found the particles inside intact mitochondria. Results of all the studies discussed revealed that the particles had never been found in the nucleus. In another study, by Bang, Ander-vont, and Vellista (1956), carried out on 41 mammary tumors of mice, both spontaneous and induced by methylcholanthrene and/or stilbestrol, characteristic viruslike particles were found in 14 of 22 tumors from mice known to carry the virus, and in 5 of 19 tumors from mice presumably free of the virus. However, mammary tumors from apparently virus-free mice were not tested biologically for the presence of the mammary-tumor-inducing virus. These investigators expressed the need for further evidence for or against identification of the characteristic particles with the virus responsible for the development of at least some mammary tumors of mice.

Fawcett (1955) described viruslike particles, similar but of somewhat larger size, in spontaneous hepatomas of virus-carrying mice, and concluded that they are the so-called milk agent or virus.

Before the results of the bioassays of the mammary tumors from apparently virus-free strain mice are presented, it should be mentioned that the relationship of both the vesicular-type and the characteristic viruslike particles to the known normal cell constituents remains unknown. The relationship between the two types of particles also appears to be a matter of

speculation only. The vesicular-type particles may be a stage in the formation of the characteristic particles or they may be an expression of the cell reaction to the presence and formation of the characteristic particles.

As already mentioned, a proper evaluation of the electron microscope observation of viruslike particles in tumor tissue can be obtained only if combined with biological tests of these tumors for the presence of the mammary tumor-inducing virus. The drawback of the bioassays is the long latent period of mammary-tumor development and the necessity of maintaining a suitable number of control test mice for observation of spontaneous tumor development in these test mice.

Preliminary results of the bioassays have already been reported (Dmochowski, 1956b, c), and a full evaluation is now possible. The incidence of spontaneous tumor appearance in the C57 Black susceptible mice has been found to be approximately 3 per cent (3 out of 90 forcibly bred mice showed mammary tumors) at an average age of 10 months, or 284 days. The incidence and appearance of spontaneous mammary tumors in forcibly bred (C57 \times RIIB)F₁ hybrid mice is shown in TABLE 1. As may be seen, 5 per cent of these mice developed spontaneous mammary tumors at an average age of 15 months, or 448 days. The results of the bioassays of the 33 spontaneous mammary tumors examined in the electron microscope must be analyzed with the results obtained in the control test mice in mind.

The results of the bioassays of the 33 tumors in the test mice are shown in TABLES 2 to 7. As can be seen in the TABLES, the C57 Black strain mice were found to be less susceptible than the (C57 \times RIIB)F₁ hybrid mice in the tests of these tumors. If an incidence of tumors in the test mice twice as large as the normal incidence of cancer is taken as a positive result in the bioassays, then 6 of the 33 tumors tested were found to harbor the mammary tumor-inducing virus. Of the 6 tumors that gave positive results in the bioassays, 4 were also found to contain the characteristic viruslike particles. Seven mammary tumors that were found to contain the particles gave either entirely negative results in the bioassays or induced only an insignificant incidence of tumors in test mice. A comparison of the results of the electron microscope studies and of the bioassays is presented in TABLES 8 to 11. As may be seen, an agreement in the results obtained by both methods was reached only in 73 per cent of the examined mammary tumors of apparently virus-free strain mice. Comparison of the results has shown sufficient disagreement to justify the conclusion that the case for regarding the viruslike particles as the mammary tumor-inducing virus has not been established in a convincing manner. Both methods of testing may not be sufficiently accurate to permit complete agreement. The presence of a latent virus unconnected with the mammary cancer must also be taken into consideration, as mice are known to carry a number of known viruses and may also carry viruses as yet unknown.

Before the next study is presented, it should be mentioned that viruslike particles have been found in a number of tumors of known virus etiology: in Shope fibroma by Bernhard, Bauer, Harel, and Oberling (1954); in Rous sarcoma by Gaylord (1955) and by Bernhard (1956), although in very small

TABLE 1
INCIDENCE AND APPEARANCE OF SPONTANEOUS MAMMARY TUMORS IN FORCIBLY BRED (C57 × RIIIb)F₁ HYBRID MICE

No. of mice set aside	No. of mice alive at earliest tumor appearance	Number of mice with tumors (according to months and days)																	Total No. of mice with tumors	Average tumor age (months/ days)	Tumor necrosis (per cent)	Average age of mice dying without tumors (months) (days)						
		Number of mice dying without tumors																										
		5	6	7	8	9	11	13	15	16	17	18	19	20	21	22	23	24					Over 24					
259	219	121	152	182	213	244	305	366	425	456	486	517	547	578	609	689	670	700	731									
		151	181	212	243	273	334	396	455	485	516	546	577	608	638	669	699	731	plus									
259	219	0/3	0	12	0	2	1	0	3	3	1	2	1	0	1	0	1/5	0/4	0/1	0	1	11/228	14	421	5	0	15	448

Controls: (C57 × RIIIb)F₁ mice.

TABLE 2
RESULTS OF THREE BIOASSAYS OF C3H α STRAIN (BETHESDA) TUMOR EXTRACTS IN C57 STRAIN MICE

Type of tumor	No. of mice injected	No. of mice alive at earliest tumor appearance (5 months, 121 days)	Number of mice with tumors (according to months and days)												Total No. of mice with tumors	Average tumor age (months) (days)	Tumor incidence (per cent)	Average age of mice dying without tumors (months) (days)
			Number of mice dying without tumors															
			9	10	13	17	18	19	20	21	22	23	24	over 24				
No. 45703	12	12															0	19/563
No. 45919	6	6	0/1	0/2	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/2		0/1	0/12	0	0	13/382
No. 46465	4	3			0/1	0/1	0/1						0/1		0/6	0	0	18/538
															0/3	0	0	
			244	274	366	486	517	547	578	609	639	670	700	731	No. dead	(months) (days)		
			273	304	396	516	546	577	608	638	669	699	731	plus				

TABLE 4
RESULTS OF FOUR BIOASSAYS OF RIIIb STRAIN TUMOR EXTRACTS IN C57 STRAIN MICE

Type of tumor	No. of mice injected	No. of mice alive at earliest tumor appearance (5 months, 121 days)	Number of mice with tumors (according to months and days)												Total No. of mice with tumors	Average tumor age (months) (days)	Tumor incidence (per cent)	Average age of mice dying without tumors (months) (days)
			Number of mice dying without tumors															
			5	6	11	13	15	17	18	19	20	21	22	23				
No. 215	14	14	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/14	0	19/563	
No. 133	4	4	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/4	0	15/426	
No. 103	14	14	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/14	0	18/533	
No. 187	7	7	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/7	0	18/546	
			121	152	305	366	425	486	517	547	578	609	639	670	No. dead			
			151	181	334	396	455	516	546	577	608	638	669	699				

TABLE 5
RESULTS OF NINE BIOASSAYS OF RIIIB STRAIN TUMOR EXTRACTS IN (C57 \times RIIIB)F₁ MICE

TABLE 7
RESULTS OF EIGHT BIOASSAYS OF C3H STRAIN (JACKSON LABORATORY) TUMOR EXTRACTS IN (C57 × RIIB)_{F₁} MICE

Type of tumor	No. of mice injected	No. of mice alive at earliest tumor appearance (5 months, 121 days)	Number of mice with tumors (according to months and days)																				Total No. of mice with tumors	Average tumor age		Tumor incidence per cent	Average age of mice dying without tumors	
			Number of mice dying without tumors																					No. dead	(months)			(days)
			5	6	7	8	9	10	11	12	13	14	16	17	18	19	20	21	22	23	24	over 24						
No. 23	32	31		0/2	0/3	0/2		0/2	1/1		0/9	0/1											1/30	11/306	3	15/434		
No. 174	31	31	0/3	1/3	2/2	1/0		0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/2	0/1	0/1	0/1	0/1	0/1	1/3	4/26	12/367	13	14/416		
No. 133	9	9							0/1				0/1		0/2	0/1		0/2				1/8	7/212	11	18/542			
No. 210	10	10				1/0	1/0	1/0		2/3	0/1	0/3	0/1	2/0	1/0		0/1	0/3	0/1	0/1	0/2	7/3	13/369	70	11/315			
No. 10	13	13						0/1	0/1	0/1	0/3	0/1			0/1	0/1	0/3	0/1	0/1	0/2	0/1	0/13	0	0	17/514			
No. 15	15	15	0/1								0/2	0/6	0/1		0/1	0/1	0/1	0/1	0/1	0/2	0/2	0/15	0	0	15/440			
No. 51	9	9						0/2	0/2	0/1			0/1		0/1	0/2	0/2	0/1	0/1	0/1	0/2	0/9	0	0	16/485			
No. 189	8	8								0/1					0/1	0/2	0/2	0/2	0/1	0/1	0/1	0/8	0	0	21/614			

TABLE 8
RESULTS OF BIOASSAYS OF MAMMARY TUMORS

C3Hf Bethesda strain	E.M. finding particles present	No. of mice alive at earliest tumor appearance (121 days)	No. of mice with tumors	Tumor inci- dence (per cent)	Average tumor age (days)	Average age of mice dying without tumors (days)
No. 45703	+	11 (12)*	0	—(0)	—	482 (563)
No. 45919 F16	+	14 (6)	1	7 (0)	152	513 (380)
No. 46465 F17	—	18 (3)	2	12 (0)	337	630 (538)
No. 45885 F17	+	21	3	15	182	577
No. 47994 F19	—	19	0	—	—	408
No. 46092 F18	—	14	0	—	—	394
No. 46752 F19	+	24	0	—	—	358
No. 47658 F18	+	28	1	4	685	551
No. 49029 F21	+	15	1	7	575	568
No. 47266 F18	—	15	1	7	602	415
No. 46918 F17	+	28	0	—	—	491
No. 46819 F17	—	21	0	—	—	560

* Numbers in parentheses refer to C57 strain test mice.

TABLE 9
RESULTS OF BIOASSAYS OF MAMMARY TUMORS

RIIIf strain tumor	E.M. finding particles present	No. of mice alive at earliest tumor appearance (121 days)	No. of mice with tumors	Tumor inci- dence (per cent)	Average tumor age (days)	Average age of mice dying without tumors (days)
No. 213 F4	—	30	—	—	—	473
No. 215 F3	—	14 (14)*	6	43 (0)	332	520 (563)
No. 133 F3	—	6 (4)	—	—(0)	—	439 (426)
No. 103 F2	—	14 (14)	—	—(0)	—	505 (533)
No. 187 F4	—	15 (7)	—	—(0)	—	476 (546)
No. 329 F4	—	12	—	—	—	538
No. 357 F4	—	21	—	—	—	539
No. 355 F4	—	19	—	—	—	484
No. 277 F3	—	11	1	9	325	523
TVI 17B	—	25	—	—	—	489
XI 11A	—	25	—	—	—	495
TVI 18B	—	21	—	—	—	506
TVI 19B	—	18	—	—	—	467

* Numbers in parentheses refer to C57 strain test mice.

TABLE 10
RESULTS OF BIOASSAYS OF MAMMARY TUMORS

C3H Jackson strain	E.M. finding particles present	No. of mice alive at earliest tumor appear- ance (121 days)	No. of mice with tumors	Tumor inci- dence (per cent)	Average tumor age (days)	Average age of mice dying without tumors (days)
No. 23	--	31	1	3	306	434
No. 174	+	31	4	13	367	416
No. 133	+	9 (18)*	1	11 (0)	212	547 (549)
No. 210	+	10 (17)	10 (3)	70 (18)	404 (485)	556 (608)
No. 10	--	13 (7)	0	-- (0)	--	507 (491)
No. 15	+	15 (8)	0	-- (0)	--	476 (544)
No. 51	--	9 (14)	0	-- (0)	--	537 (570)
No. 189	--	8 (16)	0	-- (0)	--	625 (630)

* Numbers in parentheses refer to C57 strain test mice.

TABLE 11
SUMMARY OF COMPARISON OF E.M. RESULTS AND BIOASSAYS

Bioassay result	No. of tumors examined	Agreement in results		Disagreement in results	
		Both positive	Both negative	E.M. test positive Bioassay negative	E.M. test negative Bioassay positive
Positive (including those with 10 per cent or less tumor incidence)	33	7 (21.2%)	17 (51.5%) 72.7%	4 (12.1%)	5 (15.2%) 27.3%
Positive (including only those with more than 10 per cent tumor incidence)	33	4 (12.1%)	20 (60.6%) 72.7%	7 (21.2%)	2 (6.1%) 27.3%

numbers; in Murray-Begg endothelioma by Rouiller *et al.* (1956); and also in erythromyeloblastosis of the chicken by Benedetti *et al.* (1956). In none of these tumors, however, was it possible to provide direct evidence that the viruslike particles were the agent connected with the origin of the respective tumors.

The problem of the viral origin of leukemia in mice has been the subject of extensive studies carried out by Gross (1950, 1951a, b, 1953a, b, 1954a, b, 1955a, b, c, 1956) during the past few years. Recently a confirmation of the findings of Gross was published by Woolley and Small (1956). Some aspects of Gross's observations have also been confirmed by Law (1954), Law *et al.* (1955), Stewart (1953, 1954a, b, 1955), Dulaney (1956), and by Rudali *et al.* (1956). As reported by Gross, cell-free extracts of leukemic organs of certain strains of mice (AK and C58), when inoculated into newborn mice of two other strains (C3H Bittner and C57 Brown *cd*), induced leukemia and/or parotid gland tumors. In view of these observations, studies of organs of mice from strains of mice with a high incidence of leukemia were undertaken in an attempt to establish, in the first instance, whether viruslike particles are present in these organs. Preliminary results of these studies have already been reported (Dmochowski *et al.*, 1956).

High-leukemic AKR strain mice from the laboratories of Lloyd W. Law in Bethesda, Md., and C58 strain mice from our own laboratories were used when in an advanced stage of leukemia. In all mice the cervical, inguinal, mesenteric lymph nodes, the thymus gland, and the spleen were removed whenever they showed gross pathological changes. The kidneys and the liver were also removed whenever they showed gross involvement.

The control mice consisted of the same type of tissues from young (6 to 8 weeks old) AKR and C58 strain mice free from leukemia both clinically and on post-mortem examination.

All tissues from the control mice so far examined have been found free from any viruslike particles or cellular changes that would indicate the presence of a virus.

In tissues affected by leukemia, cells in various stages of breakdown frequently have been found, as have changes in cells suggestive of the presence of a virus. In these cells, as well as in cells with very few signs of cellular damage, characteristic viruslike particles and inclusion bodies have been observed.

In all cells examined, the development of viruslike particles and the changes observed in cells appear to follow the same pattern. It may be that, with the development of viruslike particles, the appearance of inclusion bodies, the formation of virus particles in these bodies, and their release into the surrounding cytoplasm, a progressive damage occurs in the cell cytoplasm. Progressive vacuolization of the cytoplasm, destruction of the cell elements, and formation of large unidentified bodies may take place simultaneously or as a result of the appearance of viruslike particles. The complete disintegration of cells of the affected organs or tissues appears to occur frequently with the release of virus particles into the surrounding intercellular spaces.

The appearance of unaffected cells in the cervical lymph node of a leukemic mouse may be seen in FIGURE 15. The cytoplasm, the nucleus of cells, and the cytoplasmic constituents appear to be well preserved. The cells are outlined by a well-delineated cellular membrane. The ergastoplasm (composed of two parallel membranes in one of the cells) and mitochondria in two of the cells are of normal appearance. Well-defined nuclear membranes are seen surrounding large nuclei comprising large portions of the cells. Nucleoli in two of the cells are clearly evident. The ground substance of the cytoplasm is composed of small opaque particles scattered throughout the cytoplasm.

Some of the changes taking place in a cell of the thymus gland of a leukemic mouse are shown in FIGURE 16. The cytoplasm of the cell shows vacuolization, with one large, apparently unchanged vacuole near the nucleus. Some of the mitochondria are ruptured, others have lost their internal structure. The cellular membrane has disappeared, and the nuclear membrane can still be discerned.

What may be an initial stage in the formation of inclusion bodies and virus particles may be seen in one of the cells in the center of a section of a leukemic thymus in FIGURE 17. The cell membrane and the ground-substance mitochondria appear to be relatively unchanged. In three places in the cytoplasm, however, there is an aggregation or condensation of the cytoplasm that may lead to the formation of inclusion bodies. No viruslike particles are visible.

FIGURE 18, representing a section of a leukemic cervical lymph node, shows an advanced breakdown of cells. Inclusion bodies in which viruslike particles may barely be seen are present, as well as a number of dense osmophilic bodies that may represent initial stages in the formation of inclusion bodies. A number of isolated viruslike particles appear above the edge of the cells, as does a small group of particles lying between two cells. There are a number of vacuoles that may be degenerated mitochondria with dense osmophilic particles inside along their edges. Part of this area is shown enlarged in FIGURE 19. Some virus particles in which an internal structure may barely be seen are present, and also inclusion bodies with densely packed virus particles showing internal structure.

Enlarged areas of the cytoplasm of cells of a leukemic spleen are shown in FIGURES 20 and 21. In FIGURE 20 some double membranes of the ergastoplasm and some well-preserved mitochondria with cristae extending across their bodies are visible. What appear to be isolated virus particles, and also dense bodies that may represent a stage in the formation of inclusions, may be discerned. These may also be seen in FIGURE 21, in which part of the Golgi apparatus and a body of the shape of the letter "Y" with some dense osmophilic bodies are present. The Golgi apparatus is composed of several pairs of double membranes that appear to begin and terminate in the ground substance of the cytoplasm. Various degenerative forms of the cytoplasm of a cell from a leukemic thymus may be seen in FIGURE 22. At the present stage of investigation it is impossible to interpret these, but it is possible that at least some of them may lead to the formation of inclusion bodies. The

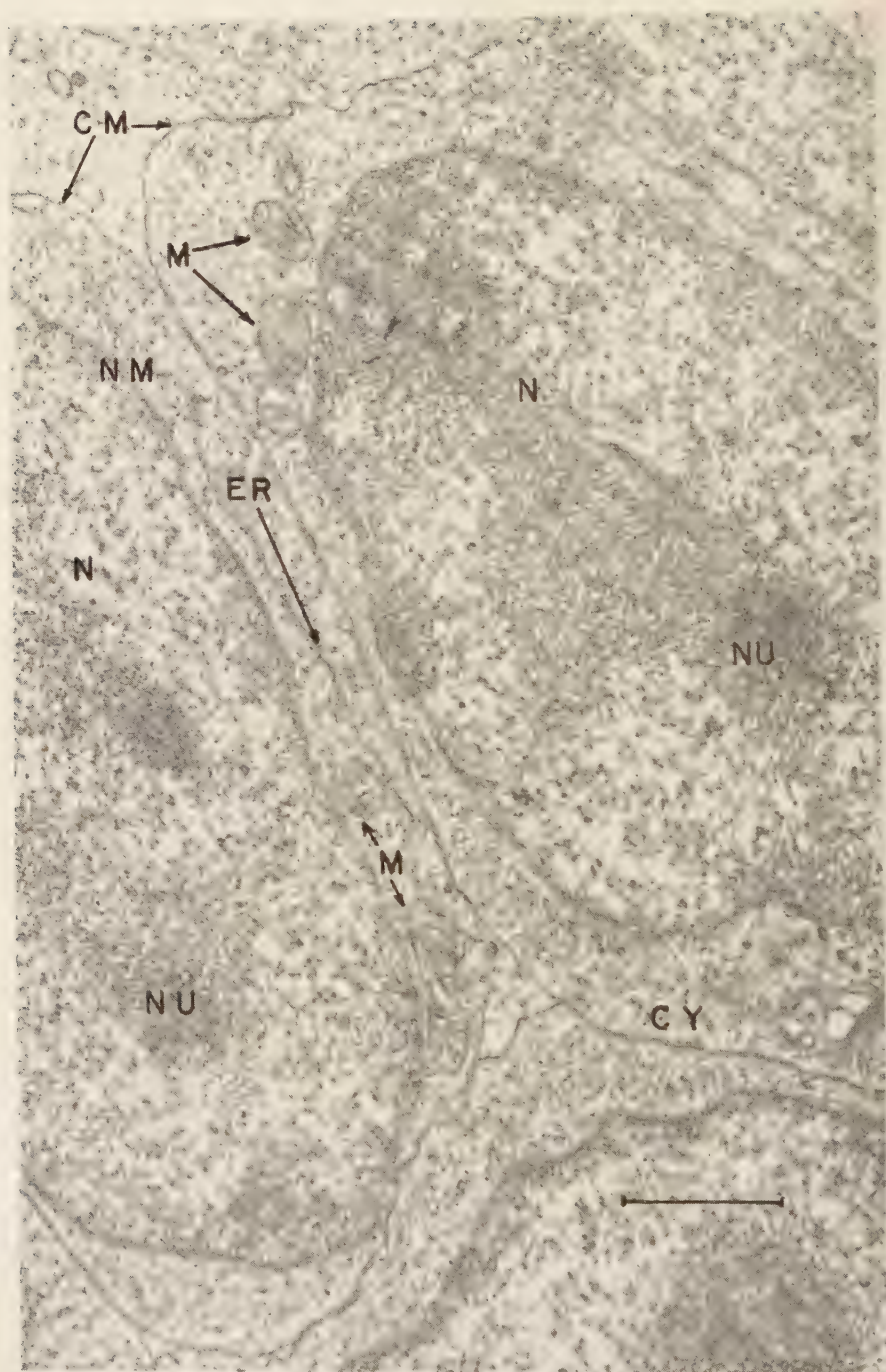


FIGURE 15. Cervical lymph node of a leukemic mouse. NU = nucleolus; N = nucleus; M = mitochondria; CY = cytoplasm; NM = nuclear membrane; ER = ergastoplasm; CM = cell membrane. $\times 20,000$.

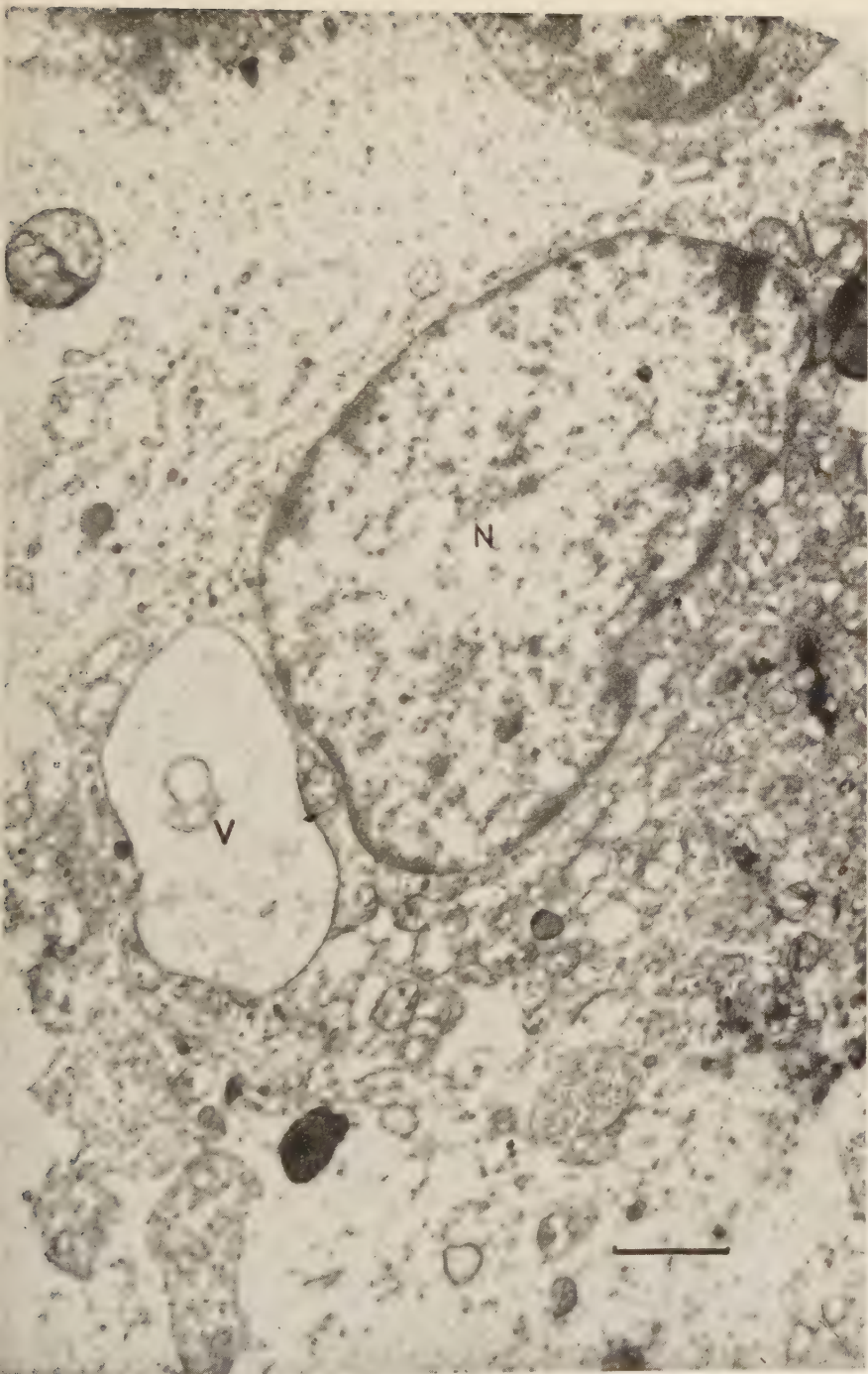


FIGURE 16. Thymus of a leukemic mouse. N = nucleus; V = vacuole. $\times 15,000$.

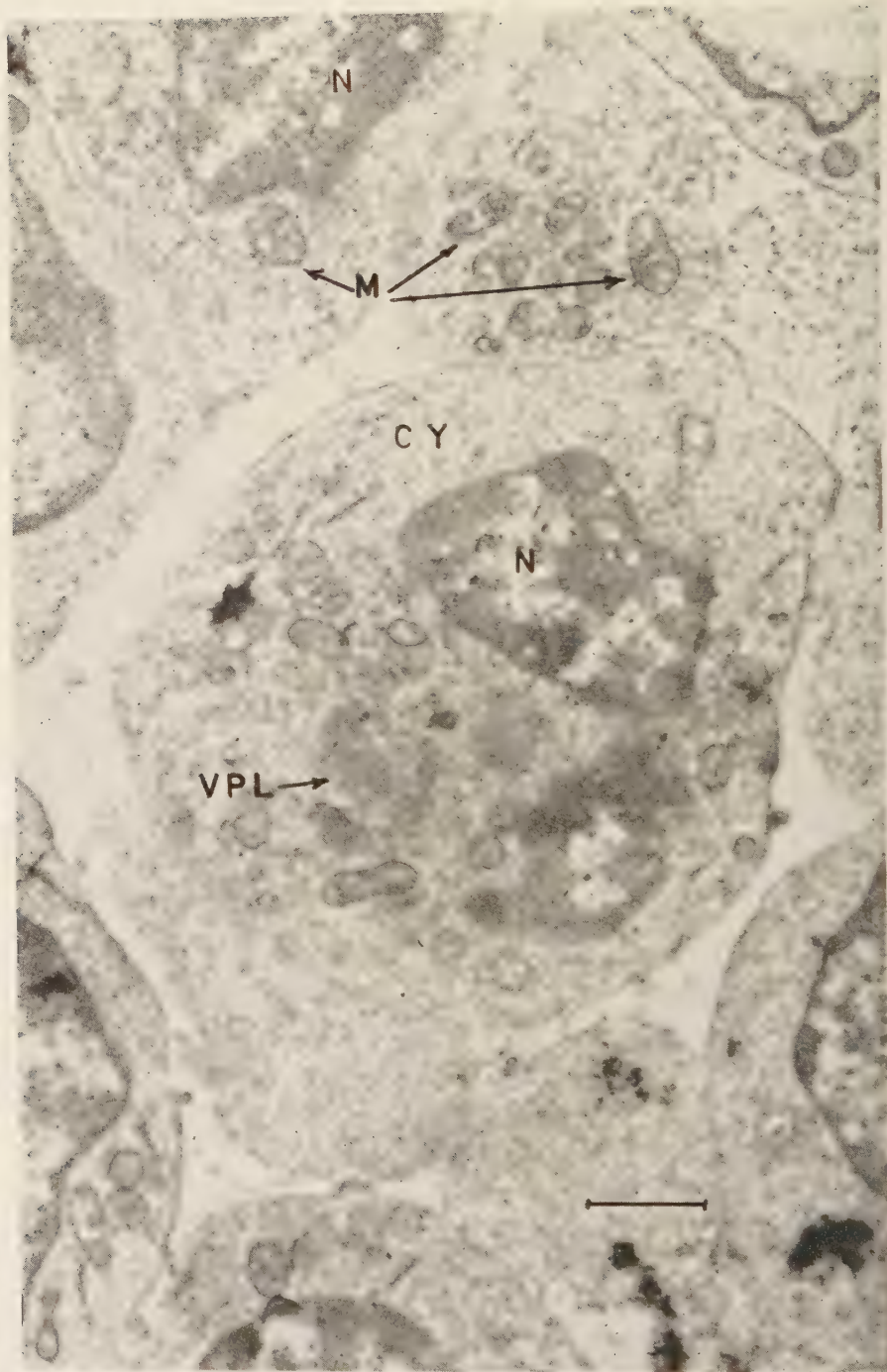


FIGURE. 17. Thymus of a leukemic mouse. N = nucleus; M = mitochondria; VPL = viroplasm. $\times 15,000$.

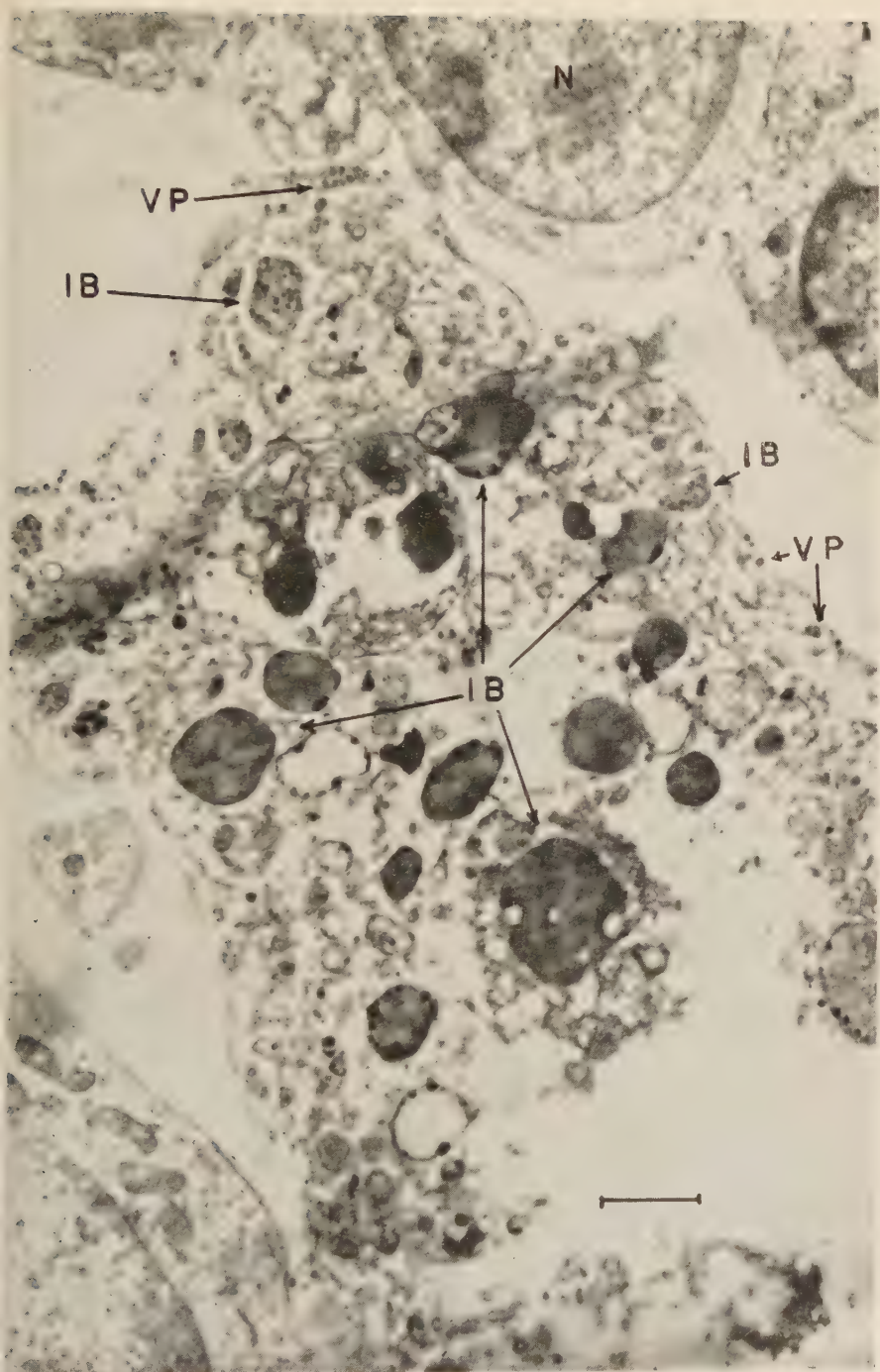


FIGURE 18. Cervical lymph node of a leukemic mouse. IB = inclusion bodies; VP = viruslike particles. $\times 12,000$.

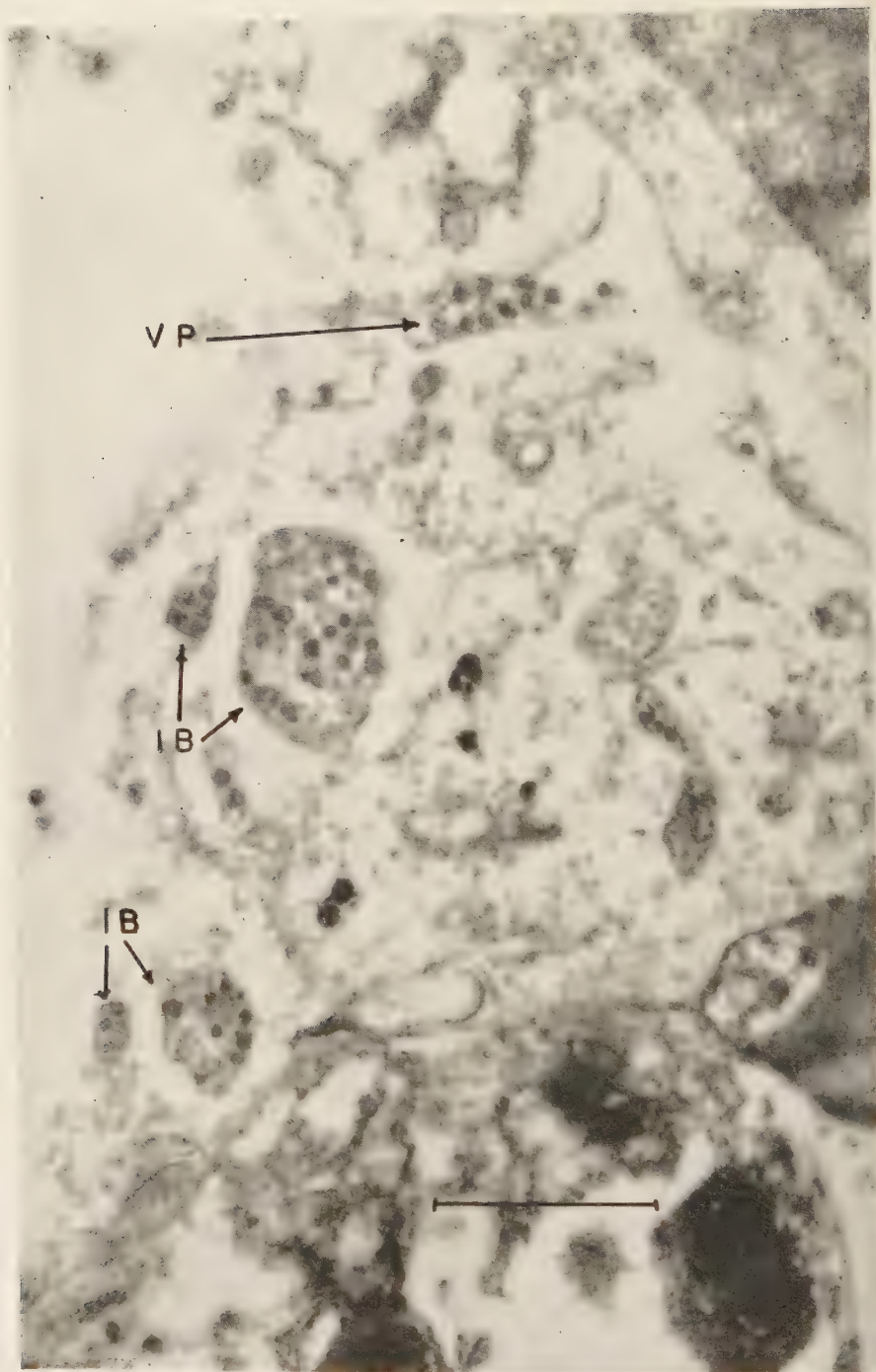


FIGURE 19. Part of the previous electron micrograph at higher magnification. IB = inclusion bodies; VP = viruslike particles. $\times 28,000$.

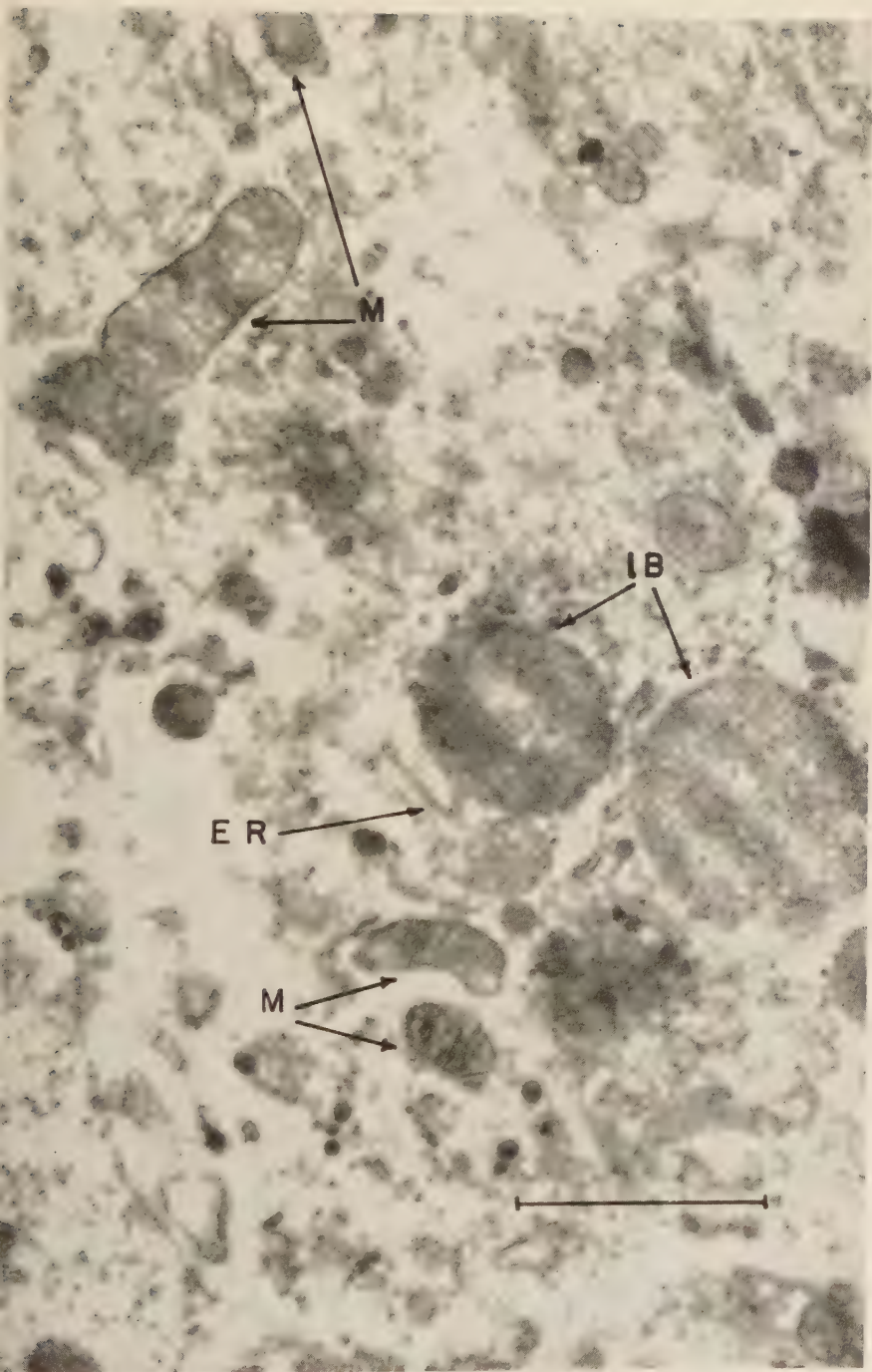


FIGURE 20. Spleen of a leukemic mouse. IB = inclusion bodies, ER = ergastoplasm; M = mitochondria. $\times 32,000$.

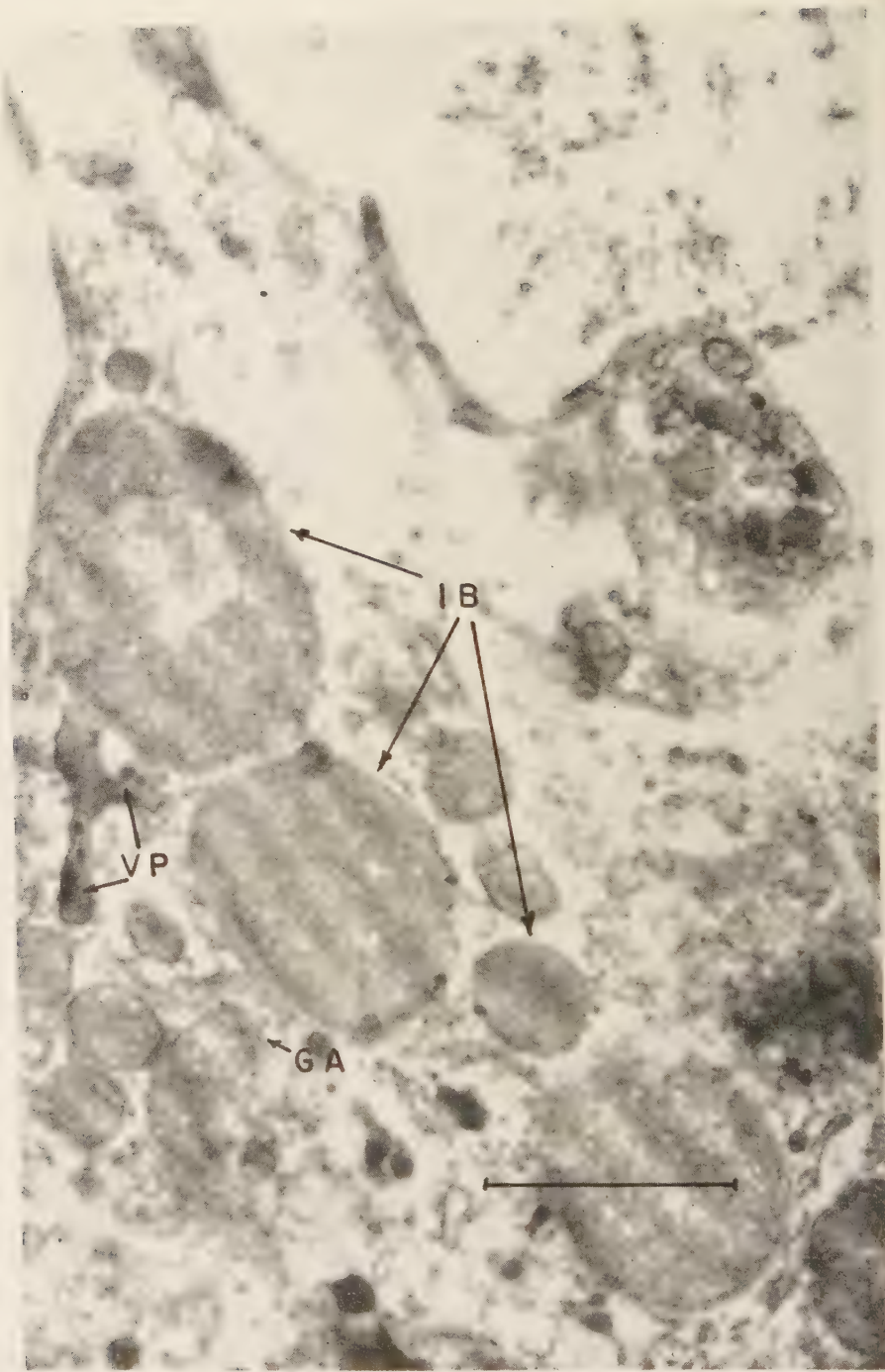


FIGURE 21. Spleen of a leukemic mouse. IB = inclusion bodies; VP = viruslike particles; GA = Golgi apparatus. $\times 32,000$.

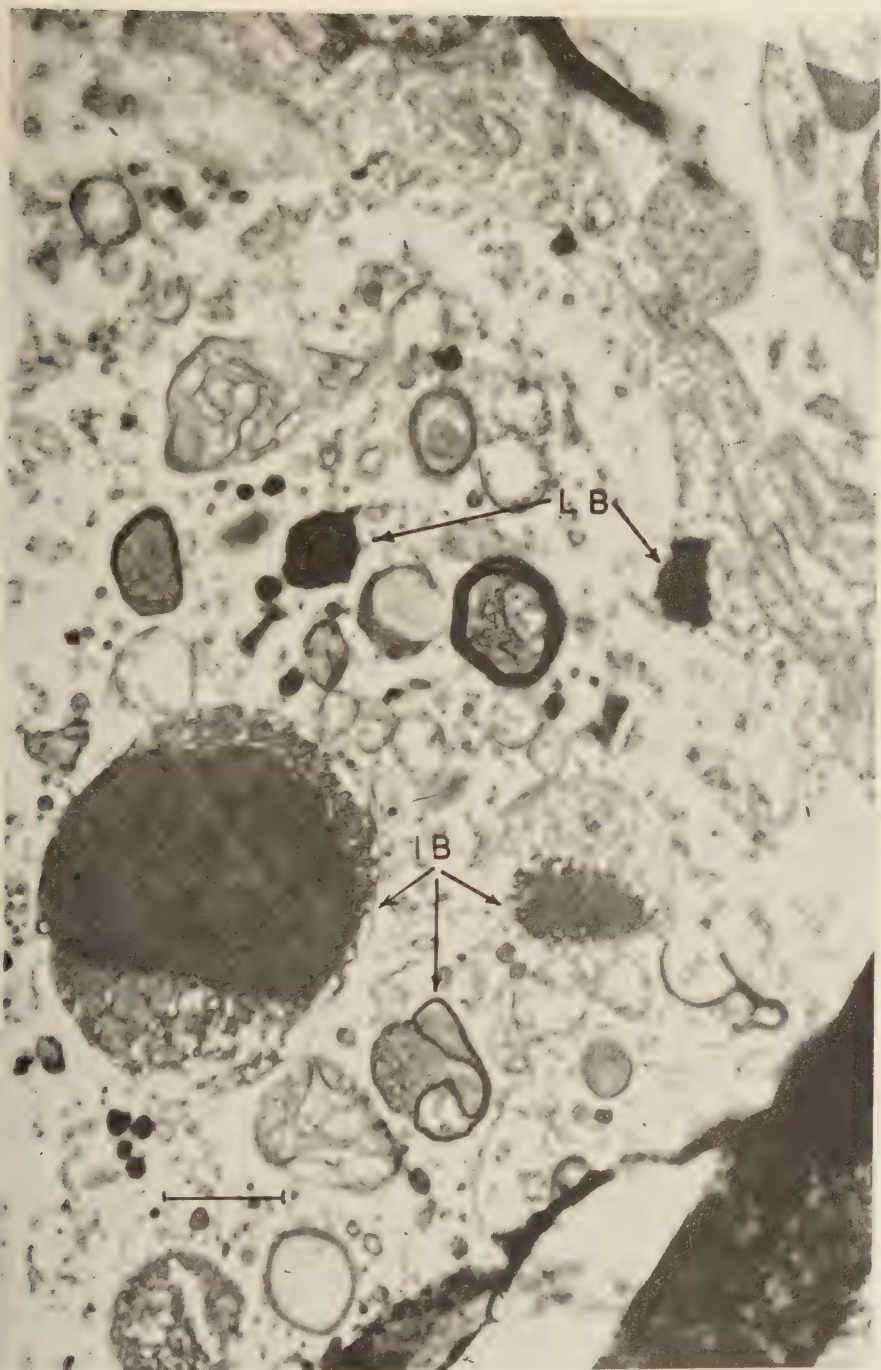


FIGURE 22. Thymus of a leukemic mouse. IB = inclusion bodies; LB = lipid bodies. $\times 15,000$.

appearance of similar bodies in the cytoplasm of cells is not always associated with striking changes in the cytoplasm of cells, as shown in FIGURE 23. In this section of a cervical lymph node, the cytoplasm of several cells may be seen with well-preserved cytoplasmic membranes, mitochondria, and double membranes of the ergastoplasm, along which are aligned Palade granules (also present, freely or in groups, in the cytoplasm). Some of the ergastoplasm appears to be extended and forming vesicles of different sizes and shapes. In one of them a row of three viruslike particles is present. Some viruslike particles are likewise present along the edge of the cytoplasm of one of the cells, in close proximity to its cytoplasmic membrane. Some inclusionlike bodies are present in the adjoining cell, with a similar but much larger body located in what may be an intercellular space.

A general picture of the appearance of virus particles and their relationship to some surrounding cells in a good state of preservation is illustrated in FIGURE 24. A very large, dense, irregular shaped body surrounded by a membrane is seen lying in a space filled by a diffuse substance. This space in turn is surrounded by what may be a cell membrane. On both ends of this body, as well as to its right, virus particles are seen in what may be either intercellular spaces or part of the cytoplasm of cells; the greater part of these cells may be lying at a different level. An enlargement of one of the virus-filled spaces (FIGURE 25) shows what may be an inclusion body of somewhat higher density, but generally similar in appearance to that of the large, centrally located body, part of which is visible. The internal structure in some of the virus particles may also be seen.

The number of virus particles encountered in the tissues of mice with spontaneous leukemia may vary from a few lying scattered inside or along the edges of the cytoplasm of cells to a considerable number present in the intercellular spaces. They may also be present in great profusion in the intercellular spaces, as shown in FIGURE 26, which represents a section of a leukemic cervical lymph node. In many of these particles an internal structure may be seen. The internal structure of the virus particles present outside the cells may be seen in FIGURE 27. In some particles one or more membranes surround a lighter zone enclosing a dense center, usually placed eccentrically. Careful study of the appearance of these particles appears to indicate that they are different in structure from virus particles found in spontaneous mammary tumors of mice. As in the case of spontaneous mammary tumors, the size of the particles in spontaneous leukemia may vary in the same section or in different sections of the same tissue. Measurement of the size of virus particles in sections from three different specimen blocks of the same leukemic tissue has given the following results: block 1, average particle size 910 Å. (varying from 670 to 1100 Å.), average size of the dense center 360 Å. (varying from 220 to 560 Å.); block 2, average particle size 1100 Å. (varying from 700 to 1400 Å.), average size of the dense center 360 Å. (varying from 210 to 420 Å.); and block 3, average particle size 1200 Å. (varying from 820 to 1650 Å.), average size of the dense center 500 Å. (varying from 270 to 690 Å.).

In the first instance, the cause of variation in the size of the virus particles

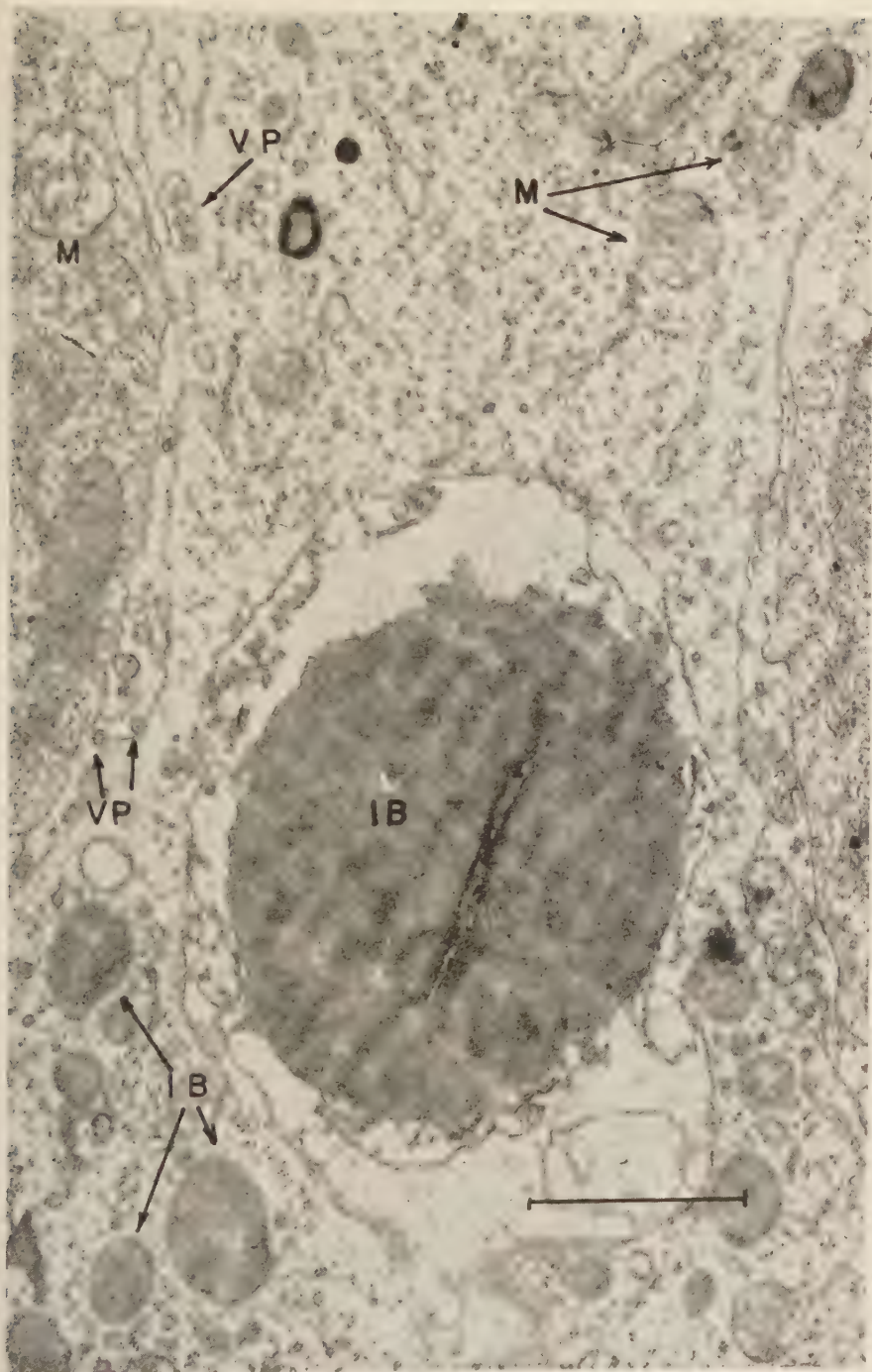


FIGURE 23. Cervical lymph node of a leukemic mouse. IB = inclusion bodies; M = mitochondria; VP = viruslike particles forming in close proximity to the cell membrane or within vacuoles of the ergastoplasm. $\times 28,000$.

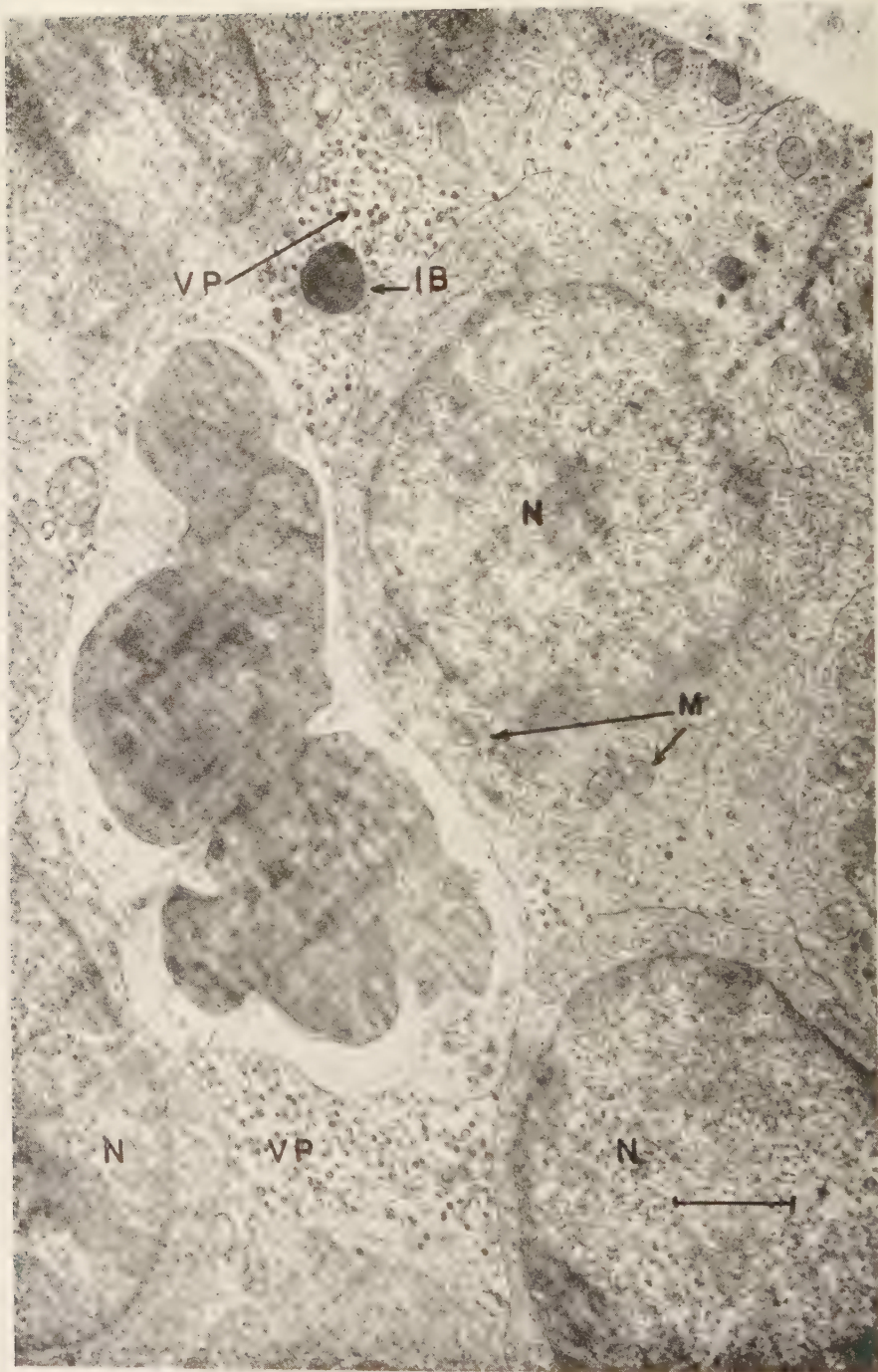


FIGURE 24. Cervical lymph node of a leukemic mouse. N = nuclei; M = mitochondria; IB = inclusion body; VP = viruslike particles. $\times 15,000$.

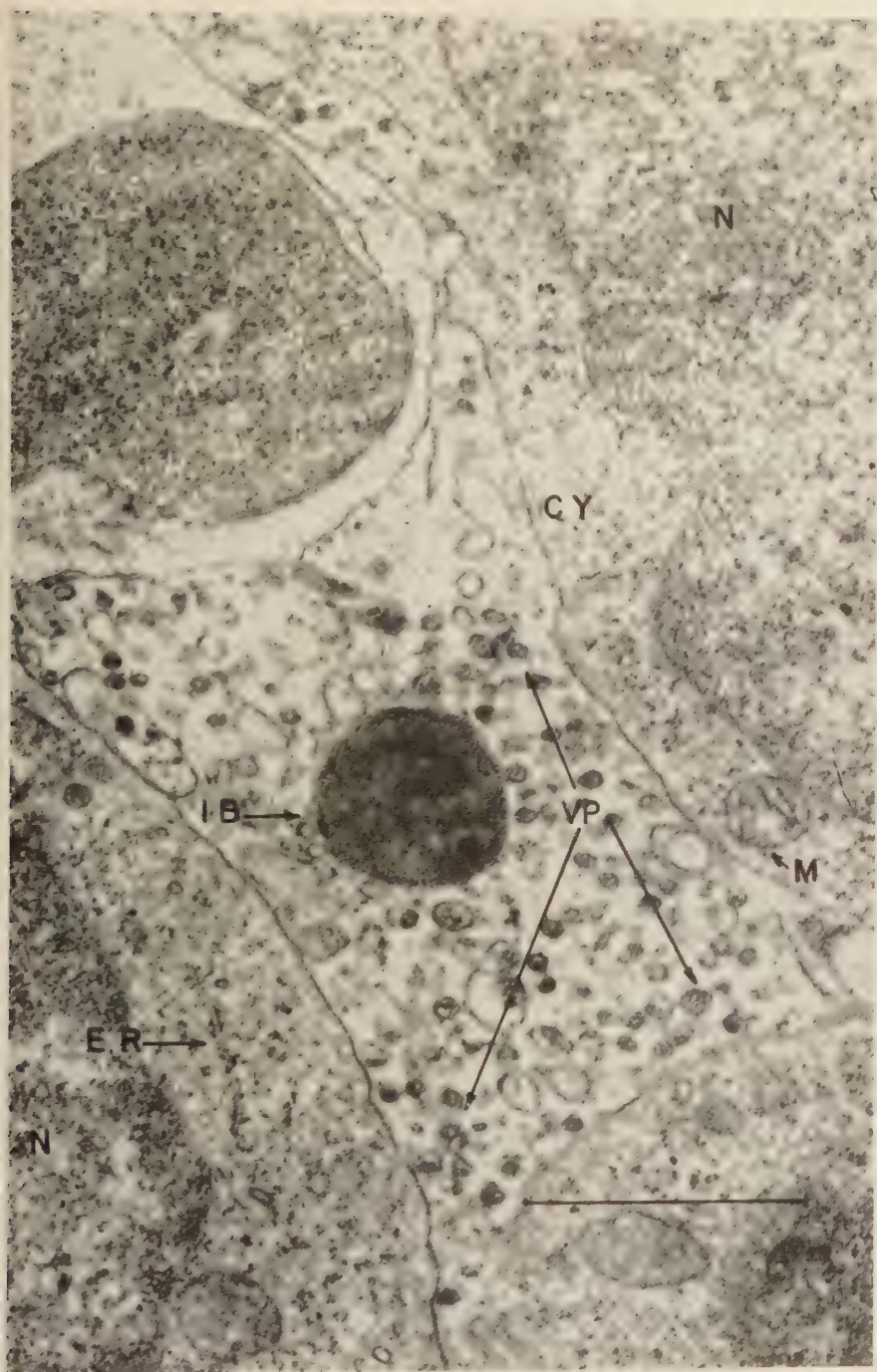


FIGURE 25. Part of the previous figure enlarged, showing inclusion body (IB); viruslike particles (VP). N = nucleus; ER = ergastoplasm; M = mitochondria; CY = cytoplasm. $\times 36,000$.

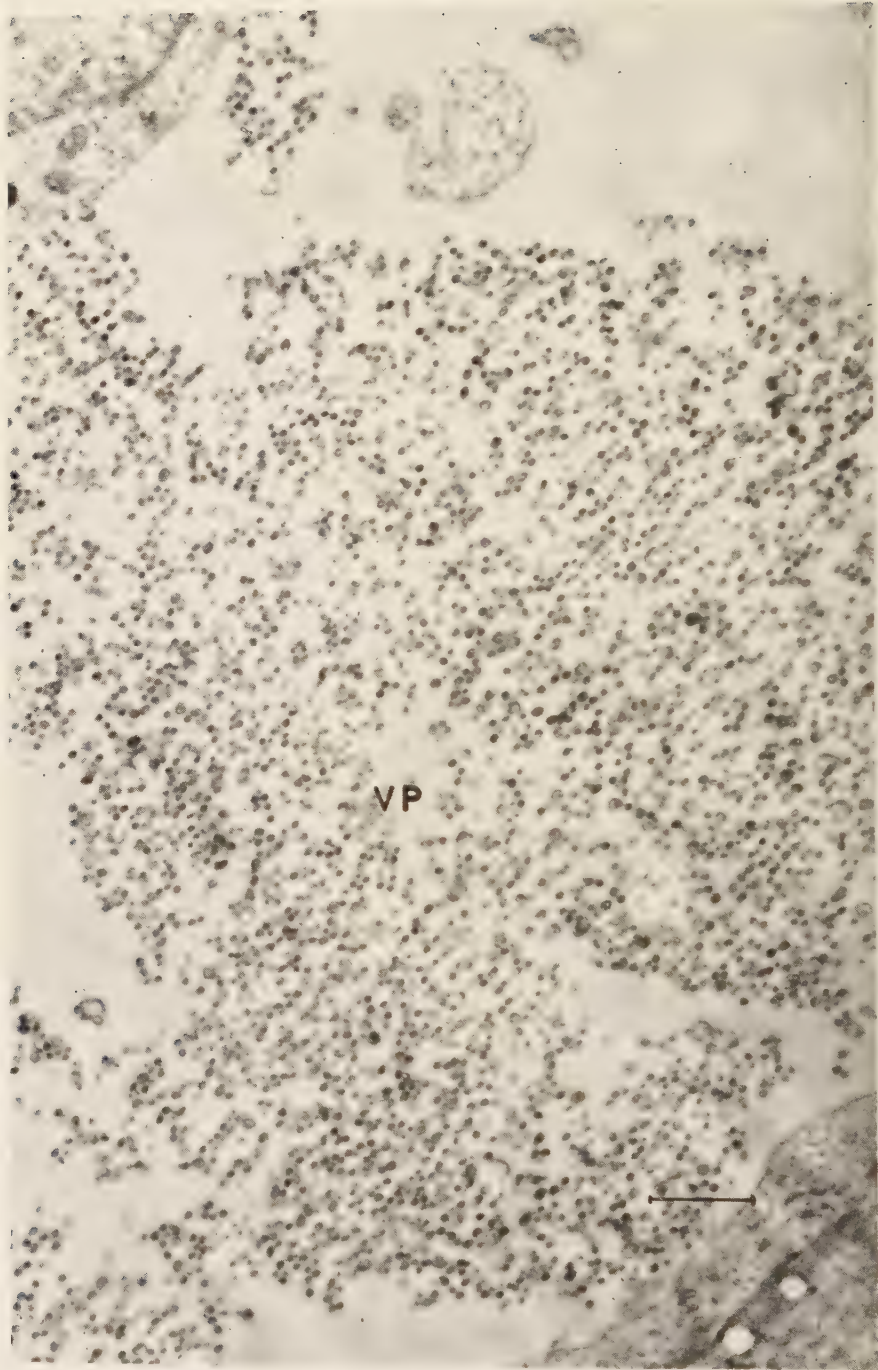


FIGURE 26. Cervical lymph node. VP = viruslike particles in great numbers. $\times 13,000$.
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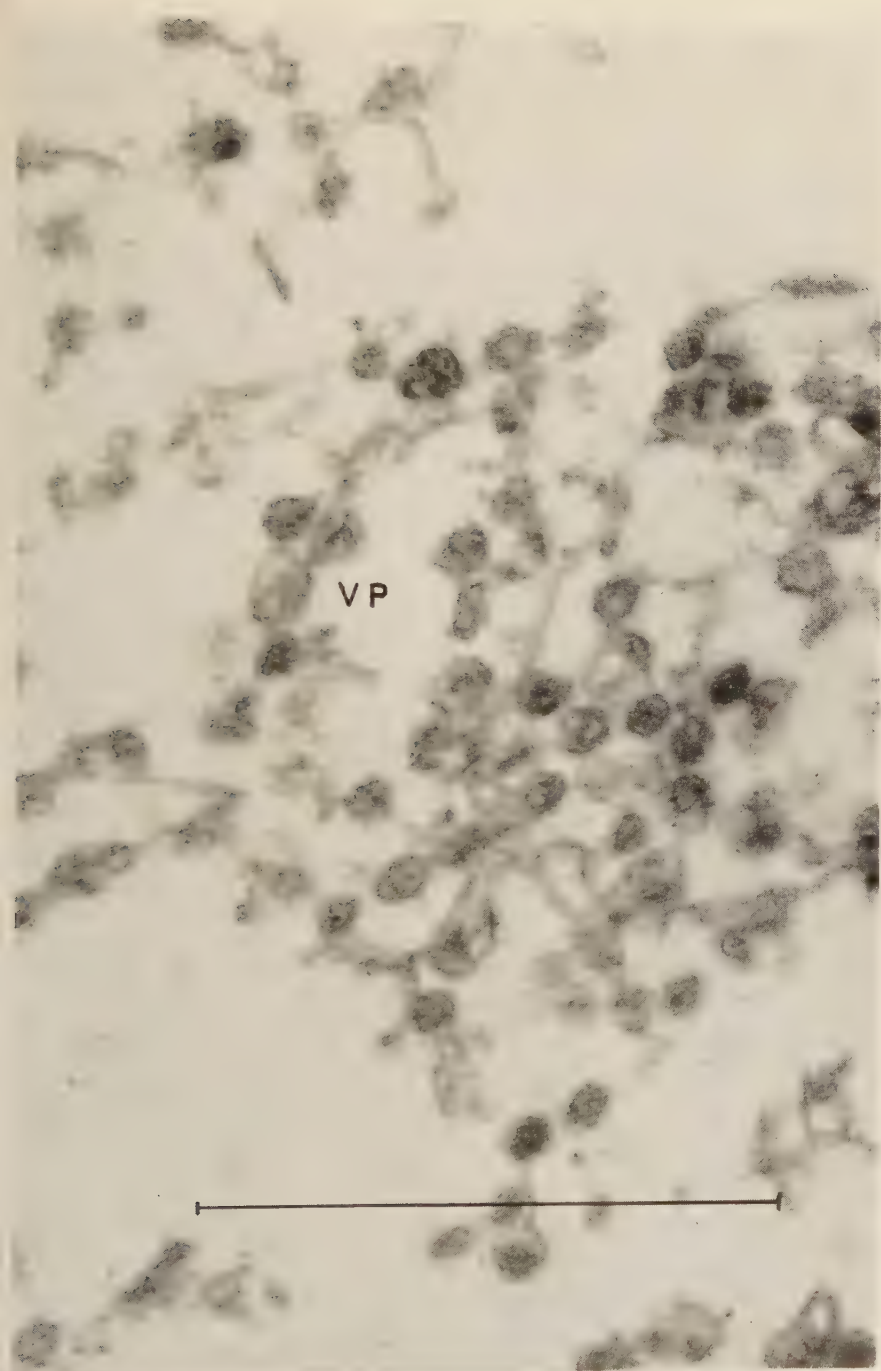


FIGURE 27. Mesenteric lymph node. VP = viruslike particles at higher magnification. $\times 75,000$.

may be the fixation with osmium tetroxide, which may differ in various areas of the same block of tissue or in different blocks of the same specimen. It is difficult, therefore, to base any conclusions on the differentiation between virus particles encountered in tissues, based on the estimation of their size, unless measurement is made of a considerable number of particles, which has not been done in the present study.

At the present stage of investigation it is not possible to form any definite conclusions regarding the relationship between the observed virus particles and leukemia of spontaneous origin in mice.

The fact that no virus particles were found in the corresponding organs or tissues of normal 6- to 8-week-old mice necessitates further study, as the particles may be present in considerably smaller numbers in such mice. The induction of leukemia in mice of a low-leukemia strain (C3H Bittner) by cell-free extracts of embryos of a strain of mice (AK) with a high incidence of leukemia was reported by Gross (1951b). It appears, therefore, that if the observed particles are in any way connected with the appearance of leukemia, they should be present in the normal organs of young mice of high-leukemia strains. It must be remembered, however, that mice carry a number of latent viruses that may tend to localize in neoplastic tissues. Many animal viruses are known to exhibit a most interesting property of preferential affinity for tumor cells in which they survive, multiply and, in some cases, destroy (Moore, 1949).

Another approach to the study of the relationship of the virus particles to the origin of leukemia in mice is an electron microscope investigation of cell-free filtrates of leukemic organs of mice that induce leukemia in suitable mice, and a similar investigation of the cells of leukemic organs from induced leukemia. Should the cell-free filtrates and the cells of induced leukemia show the presence of similar particles, a more factual relationship could be established between the virus particles and leukemia.

Gross (1956) reported an electron microscope examination of biologically active cell-free extracts of leukemic tissues of mice (C3H) with induced leukemia. Numerous spherical particles, varying in diameter from 300 to 600 or 700 Å, were present in the extracts. Characteristic indentations were observed in these particles, giving them a doughnutlike appearance. Gross stressed that it has not been determined whether these particles actually represent the leukemia agent.

In view of the obvious importance of learning whether particles similar in size and appearance are present in leukemia induced by cell-free extracts of leukemic organs of high-leukemia strain mice, a study of the appearance of cells in induced leukemia was undertaken in collaboration with Gross. Preliminary results of this study will now be reported.

The study of leukemic organs of a C3H strain mouse with induced leukemia revealed changes in the behavior of the cells similar to those observed in the cells of spontaneous leukemia. They appeared to follow a similar pattern; in addition, viruslike particles were also observed.

The appearance of cells of a leukemic thymus may be seen in FIGURES 28, 29, and 30. In FIGURE 28 a number of normal-looking cells surround a cell

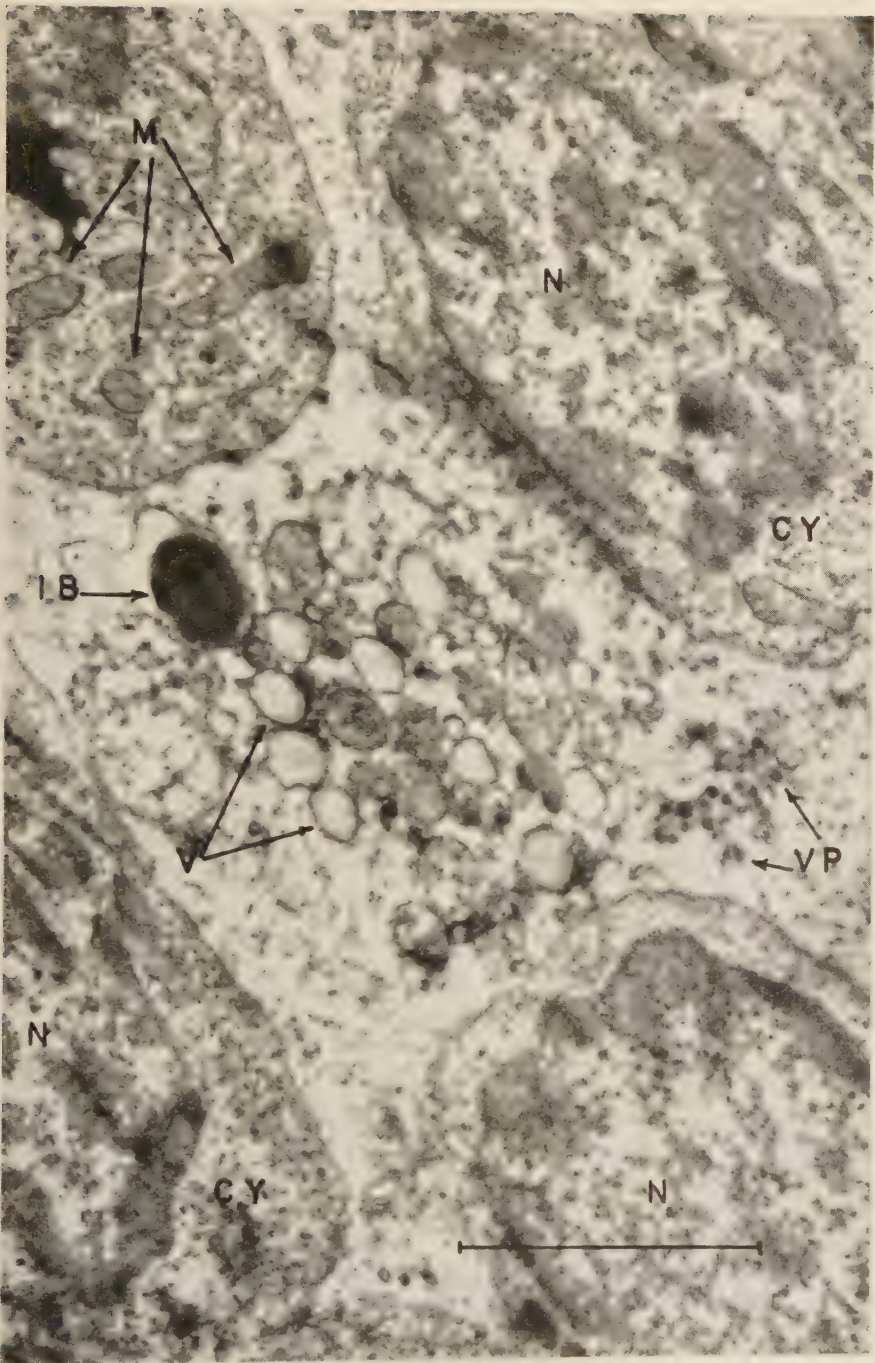


FIGURE 28. Thymus of a C3H (Bittner) strain mouse with induced leukemia by a cell-free extract of leukemic tissue. N = CY nucleus; = cytoplasm; M = mitochondria; IB = inclusion body; V = vacuoles that may be degenerated mitochondria; VP = virus-like particles. $\times 18,000$.

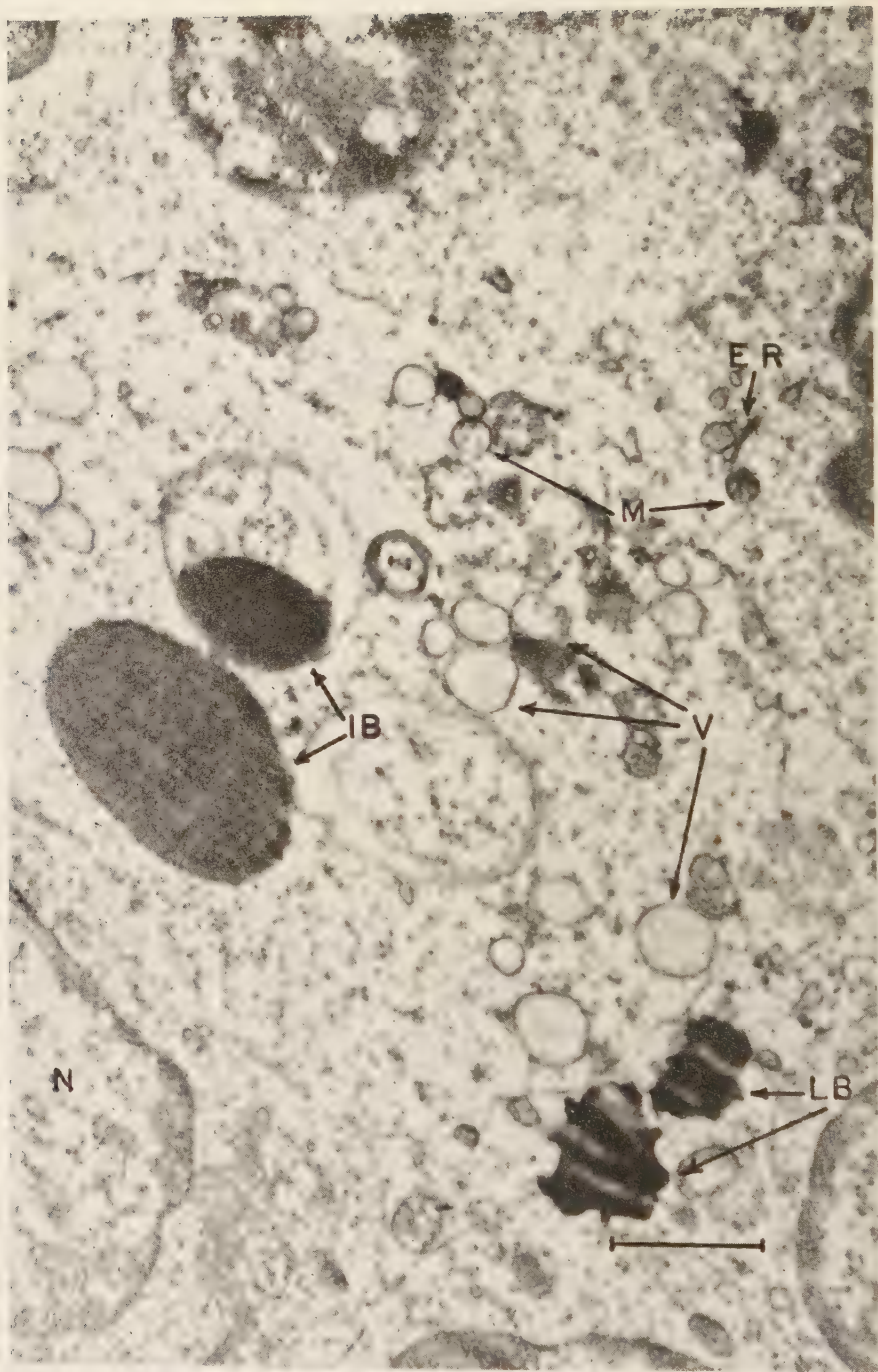


FIGURE 29. Thymus of a C3H (Bittner) mouse with induced leukemia. IB = inclusion bodies; LB = lipid bodies; M = mitochondria; ER = ergastoplasm; V = vacuoles. $\times 18,000$.

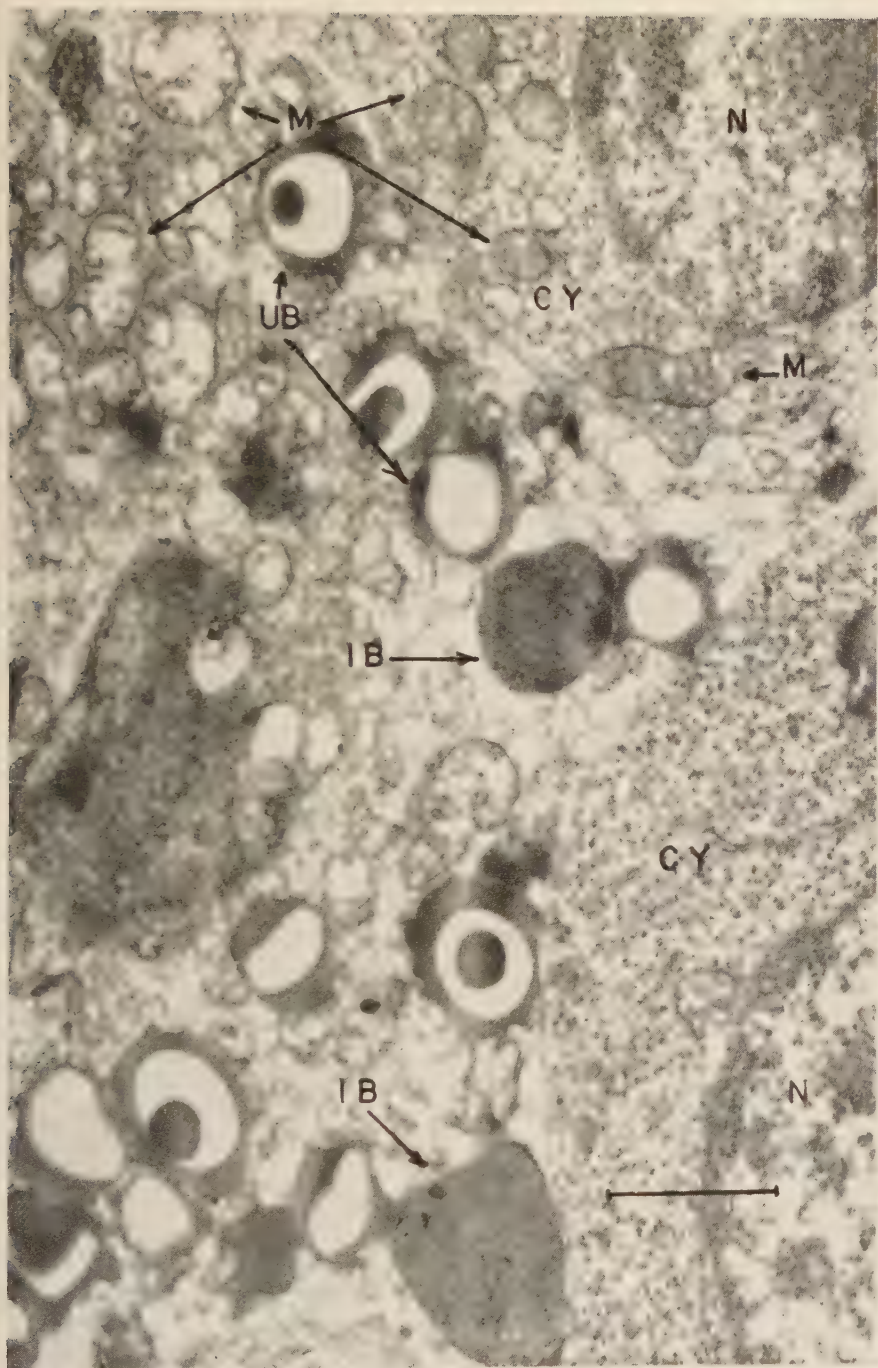


FIGURE 30. Thymus of a C3H (Bittner) mouse with induced leukemia. N = nucleus; CY = cytoplasm; M = mitochondria; IB = inclusion bodies; UB = unknown bodies, probably forms of inclusion bodies. $\times 21,000$.

in a breakdown stage. There are a number of mitochondria in various stages of degeneration, and a dense body that may be the beginning of the formation of an inclusion. In addition, a few viruslike particles may also be seen. A part of a cell undergoing breakdown is shown in FIGURE 29, with lipid bodies, dense characteristic bodies in which the formation of viruses has been observed on other occasions. Also, mitochondria in various stages of degeneration may be seen; some of these may be the vacuoles filled with a diffuse substance. In FIGURE 30 an area of cell breakdown is surrounded by two normal-looking cells of the thymus gland of the leukemic mouse. In that area mitochondria in various stages of breakdown are present, and also some dense bodies that may be the initial stages of the inclusions, as well as numerous other unknown bodies. It is difficult to say what these latter bodies are, but they may be connected with the inclusion bodies. An area of cell breakdown is shown at higher magnification in FIGURE 31. Some degenerated mitochondria and inclusion bodies may be seen. In addition, there are a number of scattered viruslike particles.

An apparently healthy cell surrounded by what may be remnants of a breakdown cell is presented in FIGURE 32. Some degenerated mitochondria are present, and also numerous virus particles in some of which an internal structure may be seen. Part of the area containing the virus particles is shown at higher magnification in FIGURE 33. At this preliminary stage of investigation it should be pointed out that the particles do not appear to show double membranes as frequently as those encountered in spontaneous leukemia. However, these particles do have a dense region of irregular appearance and are placed either centrally or eccentrically. Preliminary estimation has shown that the average size of the particle is 1250 Å. (varying from 940 to 1880 Å. in diameter) and that that of the dense region is 560 Å. (varying from 310 to 780 Å. in diameter). Additional measurements of greater numbers of particles in a number of sections of different specimens must be made before we can decide whether the size range corresponds with that of particles in spontaneous leukemia.

As already mentioned, Gross (1956) found that the size of particles in biologically active filtrates of leukemic organs varied from 300 to 700 Å. This particle size corresponds approximately with the size of the dense internal core of the particles present in spontaneous and induced leukemia. An internal structure may, however, be seen in the particles present in the cell-free filtrates. The difference in size, if the two types of particles are the same, may be due to the extraction procedures and the method used in the preparation of particles for shadow casting and electron microscopy. In the case of mammary tumors of mice, in which characteristic viruslike particles have been observed, there is a somewhat similar situation. The highest tumor-inducing activity in cell-free extracts of spontaneous mammary tumors of mice was found to be associated with particles of approximately 300 Å. average diameter (Dmochowski and Passey, 1952). In later studies the small microsomal fraction of breast-tumor tissue from high cancer-strain mice, obtained by differential centrifugation, was found to have a high tumor-inducing activity and, in the electron microscope, showed the presence

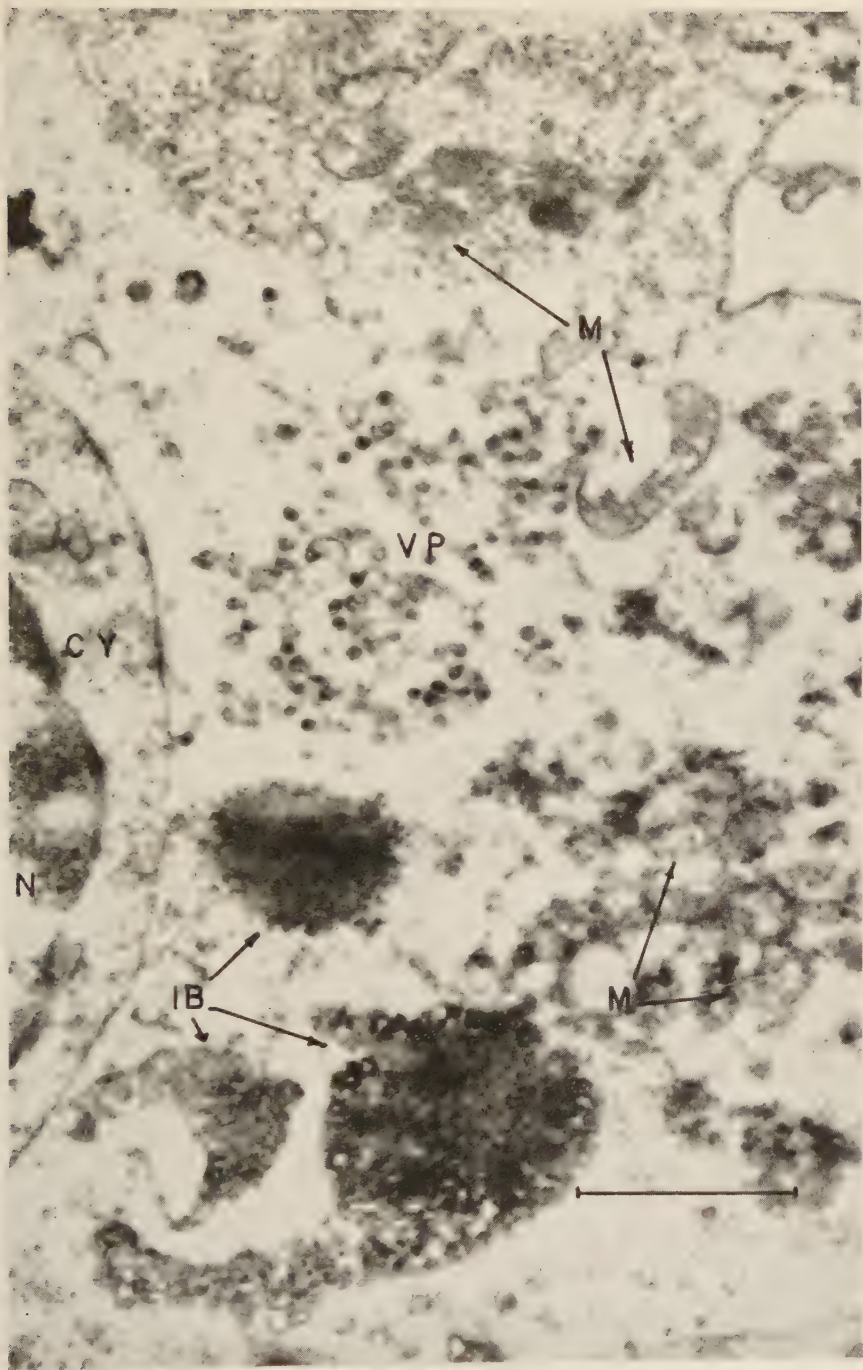


FIGURE 31. Thymus of a C3H (Bittner) strain mouse with induced leukemia. M = mitochondria; VP = viruslike particles; IB = inclusion bodies; N = nucleus. $\times 28,000$.

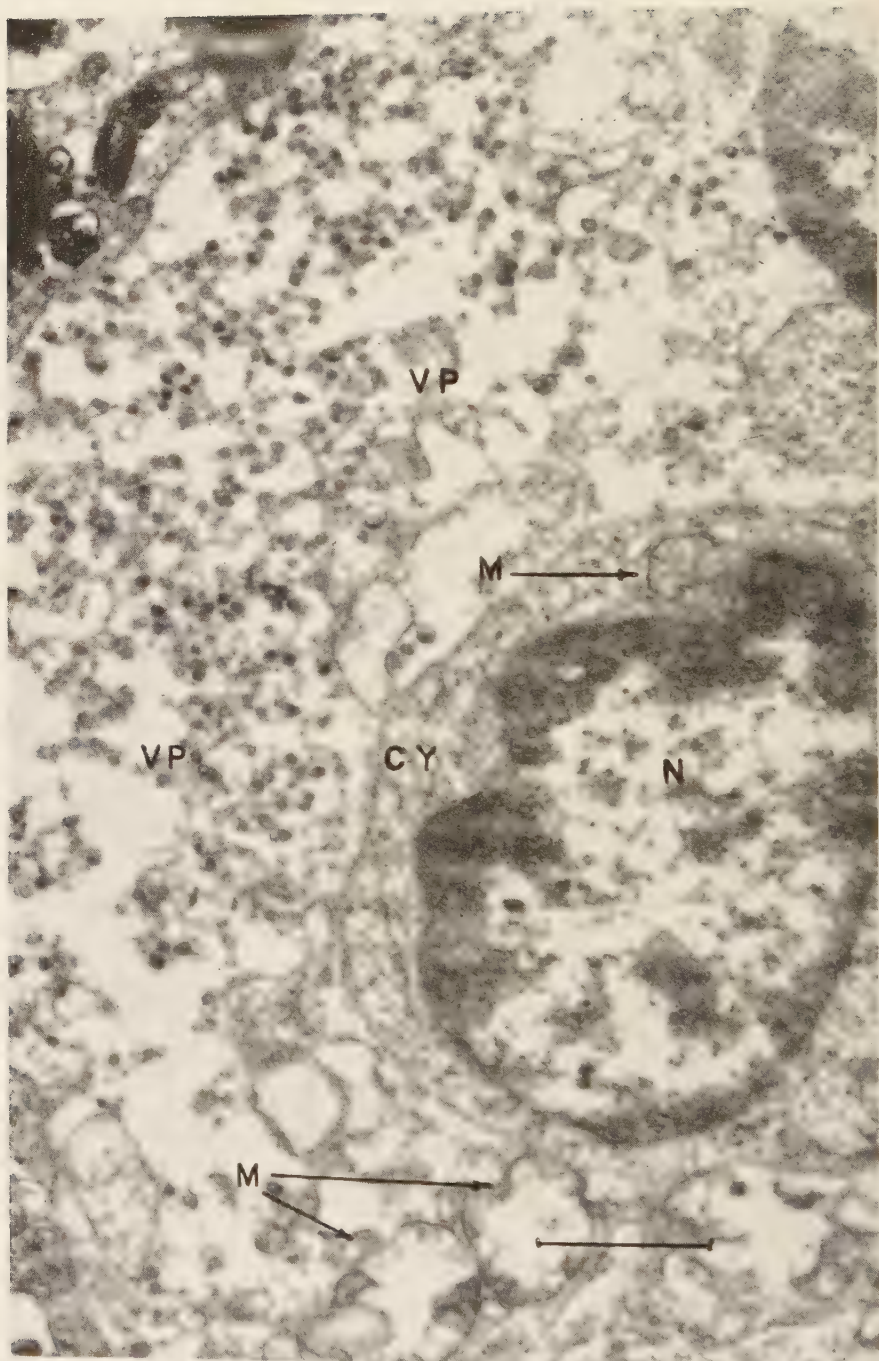


FIGURE 32. Thymus of a C3H (Bittner) strain mouse with induced leukemia. N = nucleus; CY = cytoplasm; M = mitochondria; VP = viruslike particles. $\times 21,000$.

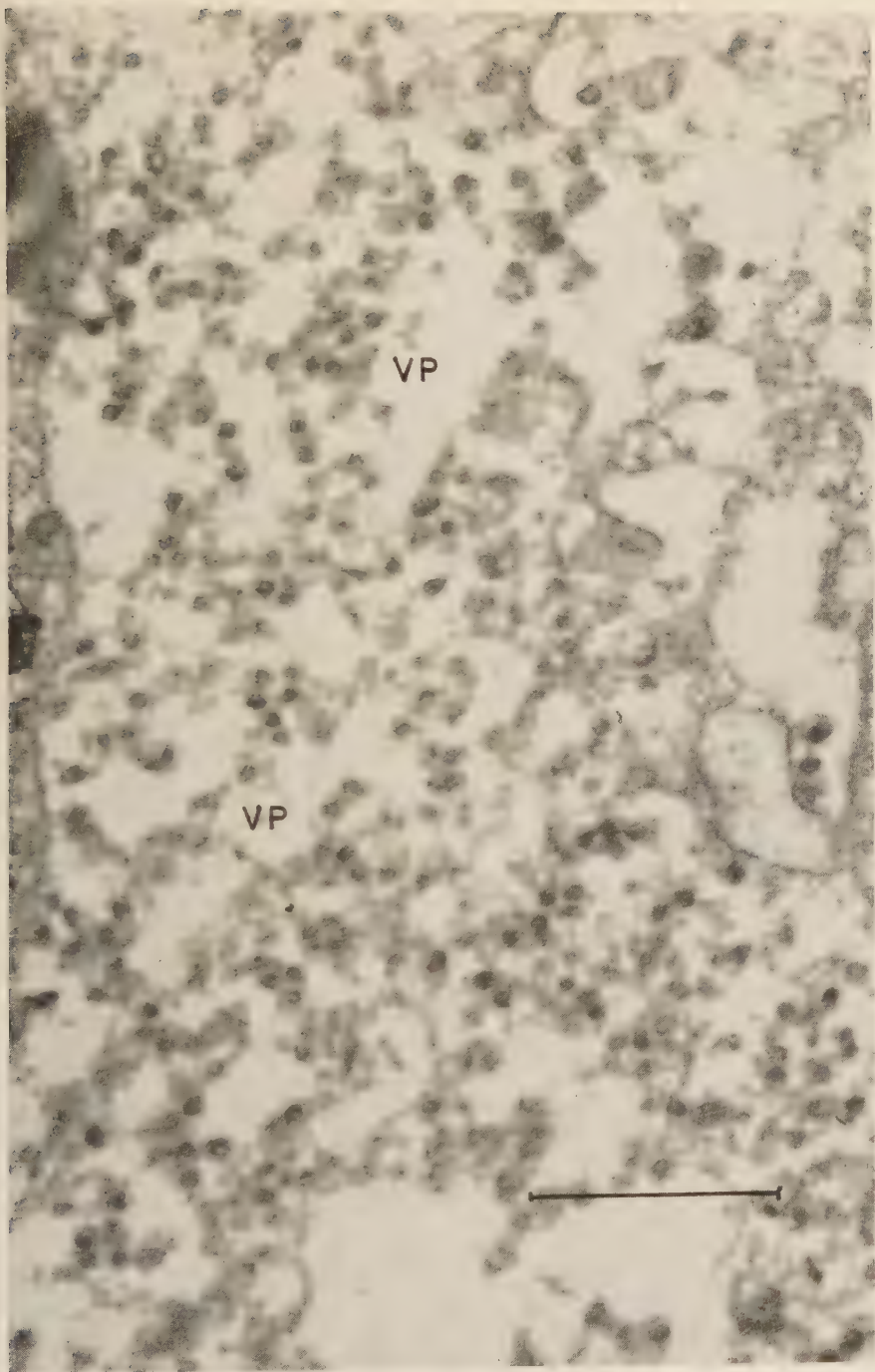


FIGURE 33. Part of an area of the preceding figure at higher magnification. VP = viruslike particles. $\times 32,000$.

of spherical particles approximately 300 Å. in diameter (Dmochowski and Haagensen, 1955). Similar particles in considerable numbers were found, however, in the microsomal fraction devoid of any tumor-inducing activity, and were obtained from spontaneous mammary tumors of mice from strains apparently free from the virus.

Additional studies now in progress employ various types of control material in an attempt to establish what relationship, if any, exists between the origin of leukemia, both spontaneous and induced, and the viruslike particles observed in organs of leukemic mice.

Summary

The results of an electron-microscope study of thin sections of 56 mammary tumors, 23 of which were from virus-carrying and 33 from apparently virus-free strain mice are presented. The electron microscope observations are compared with the results of biological studies carried out on these tumors.

Characteristic viruslike particles have been observed in 18 of 23 tumors from 3 different strains of virus-harboring mice, and in 11 of 33 mammary tumors also from 3 different apparently virus-free strains of mice.

Most of the progeny of tumorous mice from the virus-carrying strains developed a high incidence of breast cancer, although virus particles were found in only 78 per cent of the examined tumors from these mice. If a tumor incidence in the test mice (injected with cell-free extracts of apparently virus-free tumors) 3 times as high as the normal tumor incidence is taken as significant, there was a 70 per cent agreement between the results of bioassays and electron microscope studies of tumors from apparently virus-free strains of mice. In 64 per cent of these tumors the results of bioassays and electron microscope studies were negative; in 6 per cent of the tumors they were positive; in 27 per cent of cases the electron microscope findings were positive and the results of bioassays were negative; and in 3 per cent of the cases the results of the former were negative and the results of the latter were positive.

The studies, therefore, have not established the relationship between the observed virus particles and the mammary tumors of mice.

An electron-microscope study of spontaneous and induced leukemia of mice is described. In various organs from mice of two strains that developed spontaneous leukemia, virus particles as well as characteristic changes in the cells of these organs have been observed. The changes, characterized by the same pattern of cellular destruction and by the presence of the characteristic virus particles, have been observed in all organs examined.

Neither these changes nor virus particles have been found in the same organs of young 6- by 8-week-old mice of the same strains used as controls.

Similar changes of cellular destruction and virus particles of similar appearance have been observed in organs of mice of a third strain in which leukemia was induced by cell-free extracts of leukemic organs of a strain of mice with high incidence of spontaneous leukemia.

Further studies are in progress to establish the relationship between the

virus particles observed in spontaneous and induced leukemia of mice and the connection between these particles and the origin of leukemia.

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PRESENT STATUS OF NONVIRAL FACTORS IN THE ETIOLOGY OF RETICULAR NEOPLASMS OF THE MOUSE

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The recent flood of literature relating specifically to the role of cell-free agents in the etiology of "leukemia," principally in the mouse, has led to speculation and interpretations not wholly in accord with the basic knowledge gained through long, tedious, and well-controlled studies of many investigators. It appears wise, therefore, at least to attempt to bring our present knowledge into perspective.

There is now sufficient evidence at hand to indicate strongly that many different etiological factors are related to the induction or enhancement of neoplasms of reticular tissues of the mouse. Too often the general term "leukemia" is given to any and all such reticular neoplasms encountered and, dangerously, to nonneoplastic conditions that mimic superficially certain neoplastic counterparts, notably, extramedullary myelopoietic states, hyperplasias of lymphoid tissues, plasma-cell hyperplasias, and granulomatous-like lesions. This has created confusion, for it leads to the inference that the investigator is discussing the common morphologic form of lymphoma of the mouse, which is the lymphocytic neoplasm.

There is evidence, too, that leukemogenic agents evoke, not only the common thymic and nonthymic lymphocytic neoplasms, but also neoplasms of a quite different cell origin; for example, those arising from the undifferentiated stem cell, the histiocyte, the plasma cell, and other primitive unidentified cell types such as the Type B neoplasm of Dunn.¹ This being the case, the investigator should attempt to characterize as fully as possible the particular neoplasms reported.

An excellent classification of the particular forms of neoplasia of reticular tissue, with a description of the individual types, biological behavior, and occurrence in certain inbred strains and their hybrids is given by Dunn¹ (TABLE 1). Earlier reviews on the morphology and biological behavior of some of these neoplasms encountered in the mouse were given by Engelbreth-Holm,² Furth,³ and Richter and MacDowell.⁴ Each of these investigators refers also to nonneoplastic alterations in reticular tissue that simulate reticular neoplasms (see also Dunn¹).

The common form of neoplasm is lymphocytic leukemia, as found in AKR and C58 mice, following X radiation in C57BL, and following treatment with carcinogenic hydrocarbons in DBA strain mice.

Granulocytic neoplasms have been observed particularly in two strains of mice, the Rf and the F strains.^{5, 6} The diagnosis of granulocytic leukemia, however, is especially difficult because of the histological similarity to extramedullary hematopoiesis, leukemoid reactions, and myeloid metaplasias, which frequently occur. Thus, it may be necessary to supplement the

TABLE 1
CLASSIFICATION OF NEOPLASTIC CONDITIONS OF THE RETICULAR SYSTEM OF THE
MOUSE*

Cell of origin	Neoplastic condition
(1) Undifferentiated	Stem-cell leukemia
(2) Lymphocyte	{ Lymphocytic leukemia { Lymphosarcoma
(3) Granulocyte	Granulocytic leukemia (including chloroma)
(4) Reticulum cell	<i>Type A</i>
	{ Reticulum cell sarcoma
	{ Monocytic leukemia
	<i>Type B</i>
(5) Plasmacyte	Hodgkins-like lesion
	{ Plasmacytoma
	{ Plasma cell leukemia (including multiple myeloma)
(6) Tissue mast cell	{ Mastocytoma
	{ Mast cell leukemia
(7) Miscellaneous and unclassified	

* Adapted from Dunn.¹

morphologic information with as complete a biological characterization as is possible.

A disturbance involving many elements of the hematopoietic tissues, for example, was discussed by Upton and Furth.⁷ This condition may be transmitted by cell-free filtrates and indeed, without complete characterization, might have been called leukemia.

Type A and B reticular neoplasms have been observed in fairly large numbers in certain F₁ crosses^{1, 8} and in DBA 2 mice given methylcholanthrene.⁹

Plasma cell neoplasms,¹⁰ in several cases closely resembling multiple myeloma in man,¹¹ have been encountered, particularly in certain sublines of the C3H strain. The possibility of inducing these neoplasms with estrogens and radiation needs to be further investigated. Plasma cell hyperplasia has been observed in old mice of certain strains and, grossly, this picture may be easily confused with a neoplasm.

Role of Heredity in Spontaneous Leukemia

As stated previously, the common morphologic form of leukemia in the mouse is the lymphocytic neoplasm. More than 90 per cent of AKR/Lw mice develop this disease, and the mean age at death from leukemia is 8.0 months.¹² Ninety-five of 100 cases are lymphocytic, and the disease appears to be thymic in origin. The incidence of leukemia in the C58 strain is equally high; however, other morphologic forms such as the type A reticular neoplasm and granulocytic leukemia are rarely encountered.

There are several strains of mice, such as certain sublines of C3H, and the STOLI, NH, and XVII strains, in which relatively few leukemias are seen. In crosses between high- (H) and low-leukemic (L) strains, the incidence

of leukemia in the F_1 hybrid varies, and it depends upon the strains used. In our laboratory, in crosses between AKR (H) and C3H (L) strains the incidence of leukemia was 56 per cent, but it varied from sample to sample.

It is of interest to note that in most cases the incidence of leukemia was the same in reciprocal crosses, whether the high-leukemic strain was used as the mother or the father.

Two instances where F_1 offspring obtained from high-leukemic mothers ($\text{♀H} \times \text{♂L}$) showed a strikingly higher incidence of leukemia, appearing earlier in life, have been shown by MacDowell and Taylor¹³ and by Law¹⁴ not to be a positive influence contributed by the high-leukemic mother, but a resistance influence (MRF) contributed by the low-leukemic mother. This influence was shown to increase with the increasing parturition age of the low-leukemic mother. Several investigators^{3, 15, 16} have demonstrated the absence of an inciting factor, similar to the milk agent, transmitted by high-leukemic mothers of several strains of mice. A low-leukemic strain cannot be transformed to a high-leukemic strain by foster nursing. In this regard it is of interest to note the results of Fekete, who transplanted fertilized ova from AKR mice to C3H strain mothers¹⁷ and vice versa.¹⁸ The high-leukemic line retained its capacities to develop lymphocytic neoplasms, and the low-leukemic line did not develop such neoplasms beyond the extent expected.

The establishment of high-leukemic strains of mice such as AKR, C58, and F, homogeneous following continued inbreeding, led to studies by MacDowell and Richter,¹⁶ MacDowell *et al.*,¹⁹ and Furth *et al.*,²⁰ concerning the genetics of susceptibility to leukemia.

In crosses between high- and low-leukemic strains and in the production of F_2 and backcross generations, it was obvious that:

- (1) The incidence of leukemia in the F_1 was usually intermediate.
- (2) In the absence of a maternal resistance factor (MRF),¹⁶ the incidence usually was the same for reciprocal crosses: $\text{♀H} \times \text{♂L}$ and $\text{♀L} \times \text{♂H}$; that is, the capacity to develop leukemia was contributed equally by the male parent.
- (3) The phenotype of a mouse did not reveal its genotype; thus, matings of negatives gave offspring with as high an incidence of leukemia as matings of positives.

(4) An influence contributed by the parents influenced the incidence of leukemia, and the proportion of leukemias was found to be a mathematical function of the total heredity contributed by the high-leukemic strain.

Although the information obtained from ratios in the F_2 and backcross generations may indicate an influence contributed by the parents, it does not distinguish between chromosomal and nonchromosomal variables.

The problem in any genetic analysis in which heritability is not Mendelian is to determine whether the animals in a generation in which reassortment of genes is occurring are uniform or diverse in their capacities to produce leukemia. If one assumes the presence within a high leukemic strain of mice—the AKR strain, for example—of a nongenetic, cellular pathogen, this would in all likelihood be maintained in the strain at a uniform level

by inbreeding. Crossed with a low-leukemic strain, it would be reduced to lower levels.

Thus, it would be expected that all individuals in a second hybrid generation—a backcross, for example—would have uniform capacities to develop leukemia. If genes were responsible for the differences between high- and low-leukemic strains, the mice in the second hybrid generation would be a genetically diverse group, since reassortment of the genes occurs in crossbreeding, depending on the distribution of chromosomes.

The "progeny test" was used by MacDowell *et al.*¹⁹ in classifying backcross mice (high \times low leukemic strains) as to their genetic uniformity or diversity. The classification was based on the incidence of leukemia in families of mice obtained by mating backcross males to low leukemic females (and not on a plus or minus description of individual mice). Significant differences in the incidence of leukemia among families, ranging from 0 to 43 per cent, were found. If more than one gene was involved in susceptibility, a continuous variation (rather than a bimodal one) was expected and, indeed, was found. The pattern of inheritance was similar to that found in a study of lung tumors in mice.²¹

This information suggests the existence of many different genotypes among the backcross males tested, and it provides a genetic basis for susceptibility. Additional evidence was found in linkage tests. Susceptibility to leukemia was found in at least two linkage groups of the mouse.^{19, 22}

Such evidence does not, however, rule out the possibility that nongenetic variables at times play a decisive role in the etiology of leukemia. The mammary-tumor milk agent in mice has played a major role as an etiological agent in certain experimental situations, while in others it has appeared noneffective. As shown by Heston *et al.*,²³ this viruslike agent is under strict genetic control as far as its propagation and transmission are concerned. On the other hand, 40 per cent mammary carcinomas have been observed in mice of a particular C3Hf subline in which the milk agent was known to be absent.²⁴ Genetic factors are also known to govern the hormonal mechanisms concerned, and genetic differences also may be expressed through sensitivity of the mammary tissue to ovarian hormones and the milk agent.

Of three known major etiological factors (estrogenic stimulation, genetic susceptibility, and the viruslike milk agent) concerned with the induction of mammary carcinoma in mice, a combination of any two will realize the potentialities of carcinoma. However, no single factor has been shown to cause, under any set of conditions, an all-or-none difference. In delineating the etiological factors in leukemia, one must keep these experimental observations in mind.

Chemical and Physical Agents as Leukemogens

Extensive data are now available to show that three types of agents are definitely leukemogenic in mice: (1) estrogens, (2) carcinogenic hydrocarbons, and (3) radiation.

Since excellent reviews have been given recently^{25, 26} on this subject, no

attempt will be made to cover the literature, but only to point out pertinent knowledge as it relates to this discussion.

Estrogens

Marked strain differences exist in the leukemogenic response to such compounds as estrone, estradiol, estradiol propionate and benzoate, stilbestrol, and equilin benzoate. Strains C3H, CBA, and PM, particularly, show an increase in the incidence of leukemia. The induced neoplasms have been lymphocytic, apparently having their origin in thymic tissue and/or the upper mediastinal lymph nodes, as first reported by Lacassagne²⁷ and Gardner.²⁸ It must be noted that the incidence is not great, although it has been shown that estrogens augment and probably act synergistically with X rays.^{29, 30} There appears to be a dose-response relationship, with the higher doses of estrogen inducing more lymphocytic neoplasms.³⁰

Testosterone propionate significantly inhibits the induction of lymphocytic neoplasms by estrogens,³⁰ and also inhibits the spontaneous occurrence of the disease in AKR (RIL) mice.³¹ This androgenic hormone, as well as testosterone phenylacetate, is known to inhibit carcinogen-induced leukemia in DBA/2 mice.³²

Additional data supporting the role of estrogens as leukemogenic agents are to be found in studies of (1) sex differences in the spontaneous and induced disease and (2) the influence of gonadectomy (Gardner *et al.*³⁰). A particularly striking effect has been observed by Kirschbaum *et al.*³² in 6-month-old DBA 2 mice. At this age these mice resisted the leukemogenic action of methylcholanthrene. Induction of leukemia was observed, however, if the mice were gonadectomized prior to treatment with carcinogen.

The importance of the adrenal cortex and of pituitary adrenocorticotrophic hormone (ACTH) as regulators of lymphoid tissue growth and involution has been well documented. The precise role they play in the etiology of lymphocytic neoplasms is not clear.

Adrenalectomy has been found to increase the incidence of leukemia in C58 mice³³ and also to increase the incidence of the radiation-induced disease in C57BL mice;³⁴ whereas cortisone, or hydrocortisone, have been shown to depress significantly the induction by X rays of lymphocytic neoplasms in C57BL mice,³⁴ but to be without effect on the induction of granulocytic neoplasms in Rf mice.⁵ The role of the adrenal cortex in relation to other morphologic forms of leukemia has not been evaluated.

The chronic administration of pituitary growth hormone has been shown to yield localized lymphosarcomas of pulmonary origin in rats,³⁵ but has been found ineffective in mice. The neoplastic nature of these growths in rats, however, has been questioned. In mice, the pituitary appears not to be essential for the development at least of lymphocytic neoplasms, as shown by Nagareda and Kaplan³⁶ for the X-ray induced disease, and in our laboratory for the spontaneous disease in the AKR strain.⁸ Hypophysectomy had no significant influence on the incidence or the age at death from the disease.

Carcinogenic Hydrocarbons

Several strains of mice, particularly DBA, respond to carcinogenic hydrocarbons. Following skin painting or gastric instillation, 90 per cent or more of the DBA mice developed leukemia; these were predominantly, but not exclusively, thymic in origin. Further, in contrast to the neoplasms induced in this strain by fractionated radiation, numerous morphologic forms other than lymphocytic leukemia appeared following exposure to carcinogens.⁴

There is strong indication of a relationship, as with estrogens, between the dose of carcinogen employed and the incidence and latent period of the induced neoplasms.⁴⁷ These dose-response results were similar to carcinogen-induced lung tumors and sarcomas in mice.

Synergism, employing methylcholanthrene and X rays in DBA/2 mice,²⁹ has been reported.

Ionizing Radiations

Lymphomas are induced in mice by many types of radiation. Although most experimental workers have employed X rays, it is known that γ radiation of high energy, produced by atomic bomb explosions, induces leukemia, particularly granulocytic, in man and in the mouse. Fast and slow neutrons and P^{32} also are known to be leukemogenic.³⁸

Chronic radiation, as well as acute radiation, is effective. Lorenz and his co-workers,³⁹ for example, have shown that γ radiation from a radium source, 4.4 r per 8-hr. day, produced leukemia in LAF₁ mice and, interestingly enough, in 30 per cent of the C3H He strain of mice. The morphologic forms encountered were not specified.

Much of what we know concerning the pathogenesis of leukemia (particularly lymphocytic neoplasms) in mice has been obtained from experiments employing the X-ray induction of these neoplasms. The salient facts should be noted:

(1) Certain strains of mice are strikingly more sensitive to the induction of leukemia than others. The C57BL, DBA, and A strains are particularly responsive, but it must be admitted that under proper experimental conditions there is evidence for the X-ray induction of leukemia in all strains studied.

(2) The rate of induction increases with the dose employed until a plateau is reached; that is, within a certain range probability is linear with dose. It is stated that a minimal threshold of 150 r to 200 r total dose exists for the mouse, but more work in this area, especially with younger animals and with fractionated irradiation, would establish the existence of a threshold.

(3) Repeated, well-timed exposures are more leukemogenic than single exposures. Recent work by Upton and Furth,⁶ however, employing Rf strain mice, indicates that a single exposure to X rays may strikingly increase the incidence of granulocytic neoplasms.

(4) Total-body irradiation appears to be necessary. Protection of areas

of the bone marrow by thigh shielding, or shielding of the thorax, or protection of the spleen strikingly inhibits the X-ray induction of lymphocytic neoplasms.²⁶

(5) Most X-ray induced leukemias are thymic in origin and are lymphocytic; it is quite probable that many are lymphosarcomas. There are now some data to indicate that granulocytic neoplasms and the type A and B reticular neoplasms are likewise induced by X rays, or at least make an earlier appearance in the life of the animal, particularly in DBA 2 mice and in (C57BL \times A)F₁ hybrids.

(6) The leukemia-induction process is reversible within a certain time range following exposure.

(7) There is evidence of the existence of an indirect mechanism of X-ray induction, at least as it relates to lymphocytic neoplasms in the mouse. This will be discussed in detail when the role of the thymus is considered.⁴⁰⁻⁴²

General Considerations Concerning the Induction of Leukemia by These Three Classes of Agents

The following generalizations can be made concerning the induction of leukemia by estrogens, carcinogens, and radiation:

(1) Leukemogenic activities of these various agents appear to be fairly strain-specific. These agents are not equally effective on mice of all strains.

(2) Synergism has been observed in certain situations with combinations of agents at subthreshold levels.

(3) Within certain limits, the incidence of induced leukemia is proportional to the total dose given.

(4) The induced neoplasms are strikingly similar morphologically and are for the most part lymphocytic in origin. Probable exceptions to this generalization have been mentioned.

Role of the Thymus in the Etiology and Pathogenesis of Lymphocytic Neoplasms

The role of the thymus must be considered in any discussion of the etiology and pathogenesis of lymphocytic neoplasms in the mouse.

Total removal of thymic tissue in most high-leukemic strains of mice almost completely overrides the potentialities of developing leukemia. This occurs in the spontaneous or induced disease whether or not the thymus is the primary site of origin of the disease; it has been observed in the AK, AKR, DBA, C57BL, and C58 strains.⁴³⁻⁴⁶ In special cases, however (for example in the C3H-FG sublines discussed later, in which other morphologic forms of reticular neoplasms are frequent), thymectomy has no, or little, influence.

The presence of a thymic graft in thymectomized animals partially restores the potentiality of developing lymphocytic neoplasms. Paradoxically, in many cases the grafted thymic tissue apparently does not supply the progenitor cells of the neoplasm.

Autologous or isologous grafting into DBA 2 mice, followed by methyl-

cholanthrene administration, restores the host potentiality for developing lymphocytic neoplasms.⁴⁵

Kaplan and Brown,⁴⁷ employing C57BL strain mice, reported that, in thymectomized mice given fractionated X radiation and a nonirradiated thymic graft as late as eight days after irradiation, lymphocytic neoplasms developed. The origin of the neoplasms was in the grafted tissue.

These results indicated to these authors that the initiation of leukemogenesis did not require actual exposure to radiation, and thus established "the existence of a completely indirect mechanism of induction of lymphoid tumors in systemically irradiated mice."

By employing genetically labeled thymic grafts, an attempt was made in our laboratory⁴⁸ to study in more detail the relationship of the grafted thymus to this postirradiation influence. The thymic graft was obtained from one of the parental strains of a hybrid mouse. In this case the parental thymus was successfully maintained in the compatible but genetically different host. The Kaplan subline of the C57BL strain was crossed with A He strain mice; each of these strains carried different H-2 alleles, thus assuring that the F_1 hybrid mouse would have strong tissue antigens possessed by neither parent strain. Although progressively growing neoplasms from either parental strain were expected to kill (C57BL \times A) F_1 hybrid mice, such neoplasms arising in tissues of these specific F_1 mice were not expected to grow progressively in either A or C57BL mice.

As a control study, neoplasms originating in the C57BL and A strains, and in (C57BL \times A) F_1 mice, were transplanted into the two parental strains and the F_1 hybrids to establish (1) the incompatibility of the F_1 tumors in the parental strains and (2) the compatibility of parental neoplasms in the F_1 hybrid. These neoplasms included 2 pulmonary tumors of A strain origin, 4 X ray-induced lymphocytic neoplasms that arose in intact C57BL mice, and 7 of the X ray-induced lymphocytic neoplasms originating in (C57BL \times A) F_1 hybrid mice. The results of these experiments showed precisely what was expected according to the genetic laws of transplantation. Parental tumors grew progressively in the strain of origin and in hybrids involving the parental strain, whereas the F_1 lymphocytic neoplasms grew progressively only in the genotype carrying the essential antigens of both parental types, the F_1 hybrid.

TABLE 2 gives the results of this experiment, details of which have been published. Twenty of the 32 intact, irradiated mice developed lymphocytic neoplasms. Total thymectomy completely prevented the appearance of lymphocytic neoplasms. The gross appearance of the 20 induced neoplasms in the intact, irradiated controls revealed that the thymus was markedly involved in 18 of these mice. The neoplasms listed in the thymectomized, grafted group represent those that arose in thymic grafts. In these cases the first gross evidence of lymphocytic neoplasm appeared as progressive enlargement of the graft.

Lymphocytic neoplasms arising in C57BL thymic grafts at 5 and 5½ months grew progressively in C57BL and (C57BL \times A) F_1 hosts. In transplant, these neoplasms behaved as C57BL in origin. In contrast, lympho-

TABLE 2
LYMPHOCYTIC NEOPLASMS IN (C57BL \times A)F₁ MICE FOLLOWING FRACTIONATED IRRADIATION

Group	Total No.	Lymphocytic neoplasms (month)									Number of lymphocytic neoplasms*
		4	5	6	7	8	9	10	11	12	
I, intact.....	32	0	2	3	5	6	1	2	1	0	20
II, thymectomized.....	47	0	0	0	0	0	0	0	0	0	0
III, thymectomized: plus thymic graft.....	75	0	4	0	1	5	3	1	0	0	14

* Neoplasms at the site of the C57BL thymic graft.⁴⁸

cytic neoplasms arising later in C57BL thymic grafts (at 7 to 10 months) grew progressively following transplantation only in (C57BL \times A)F₁ hybrids. These neoplasms indicated their origin from F₁ cells, although the site of origin was from a C57BL thymic graft. Thus, by employing a system of genetically marked thymic grafts, it is clearly possible to differentiate between nonexposed cells of the thymic graft and exposed cells of the host that might populate the graft.

A similar type of experiment, employing AKR thymic fragments grafted into (C3H \times AKR)F₁ mice,⁴⁹ has been reported from this laboratory. In this situation the compatible graft (AKR), in the compatible but genetically different host (AKR \times C3H)F₁, underwent a change in the thymic lymphocyte population that was observed histologically and was later confirmed by transplantation studies. These fragments became neoplastic and, upon transfer, did not grow progressively in AKR mice (the strain of origin of the thymic tissue), but grew only in the specific F₁ recipient mice used.

In the study using a marked thymic graft (C57BL) in a compatible but genetically different host, it was possible to show that the postulate of Kaplan and Brown¹⁷ was, in part, correct. That is, cells of C57BL origin never exposed to X radiation did give rise to lymphocytic neoplasms, but only in 4 of 12 cases. Thus, this situation represented only part of the complete picture. A greater number of grafts that developed into lymphocytic neoplasms developed from cells derived from the irradiated host animal.

It is possible that in this experimental situation the thymus behaved as do certain embryonic tissues, that is, by direct tissue interaction. Thus, the grafted thymus represented a "sphere of influence." A similar type of action, employing the (C3H \times AKR)F₁ hybrid mouse, has been demonstrated in other experiments from this laboratory. In these experiments⁴⁹ it was found that AKR thymus was a source of lymphocytic neoplasms in this hybrid, while the equally compatible C3H thymus was not. The sphere of influence appeared to be genetically determined and was specific to certain strains. Kaplan *et al.*⁵⁰ also have shown that C57BL, but not

TABLE 3

LYMPHOCYTIC NEOPLASMS IN HYBRID MICE RECEIVING THYMIC GRAFTS FROM SUSCEPTIBLE AND REFRACTORY DONOR STRAINS

	(C57BL × C3H)F ₁ *		(C3H × AKR)F ₁ †	
Source of implant.....	C57BL	C3H	AKR	C3H
No. of mice.....	54	58	103	25
No. neoplasms‡.....	14	2	57	0
Per cent neoplasms.....	26	3%	55.3%	0%

* Data adapted from Kaplan *et al.*⁴¹

† Unpublished observations of Law.

‡ Lymphocytic neoplasms appearing at the site of the thymic graft.

C3H thymic fragments, became lymphosarcomatous in the irradiated F₁ mouse. These results, along with our previously unpublished observations, are shown in TABLE 3.

It is obvious that genetic susceptibility to the development of lymphomas is expressed as an intrinsic property of thymic tissue. Similar findings have been reported for the target tissues concerned with lung adenomas^{51, 52} and postcastrational adrenal tumors.⁵³

It may be stated in summary that:

(1) The thymus appears to be a *sine qua non* for the development of lymphocytic neoplasms in certain inbred strains of mice. The part thymic tissue plays in the etiology of other morphologic forms of leukemia remains to be determined. Transfer of thymic tissue to thymectomized mice restores the ability to develop lymphocytic neoplasms.

(2) Paradoxically, in many cases the grafted thymic tissue does not supply the progenitor cells of the neoplasm, but has the characteristics of a sphere of influence from which the neoplasm arises. Also, however, and particularly in radiation-induced lymphomas, these neoplasms arise from nonirradiated homologous thymic grafts, indicating a postirradiation conditioning of the animal. In our laboratory, thymic tissue grafted as late as 28 days after the last X-ray treatment gave rise to lymphocytic neoplasms.

(3) The genetically determined susceptibility to lymphocytic neoplasms is expressed as an intrinsic property of thymic tissue.

Low-Leukemic Strains of Mice

Ample observations and data have been compiled to indicate that several strains of mice rarely develop neoplasms of the lymphatic, reticuloendothelial, or hematopoietic systems. These include particularly STOLI, NH, CBA, C57 Black, BALB, XVII, and certain sublines of the C3H strain. It has been observed that genetic divergencies have occurred among sublines maintained in different laboratories. MacDowell *et al.*⁵⁴ have observed, for example, a high incidence of leukemia in their BALB strain, occurring extremely late in life. In other laboratories this strain behaves and has been referred to as low-leukemic.

Many sublines of the C3H strain have been developed from the original line started by Strong in 1920. The striking morphologic differences (particularly vertebral and skeletal⁵⁵) in β -glucuronidase activity,⁵⁶ and in histocompatibility patterns⁵ among the different sublines have been studied and may be ascribed to genetic differences. A genealogical table of many of the sublines now in existence was published by McLaren and Michie.⁵⁵

Certain sublines of the C3H strain have been employed as test mice in experiments designed to investigate the possible virus etiology of leukemia. As a whole, the strain has been referred to as low-leukemic or, erroneously, as nonleukemic.⁵⁷ Among the An, He, and Lw sublines maintained at NCI, the incidence of leukemia varies from sample to sample, but has been found to be as high as 10 per cent.¹ Occasionally, generalized lymphocytic neoplasms make their appearance early in the life of the animal.⁵⁸ FIGURE 1 is a genealogical chart of some of the C3H sublines especially referred to in this discussion.

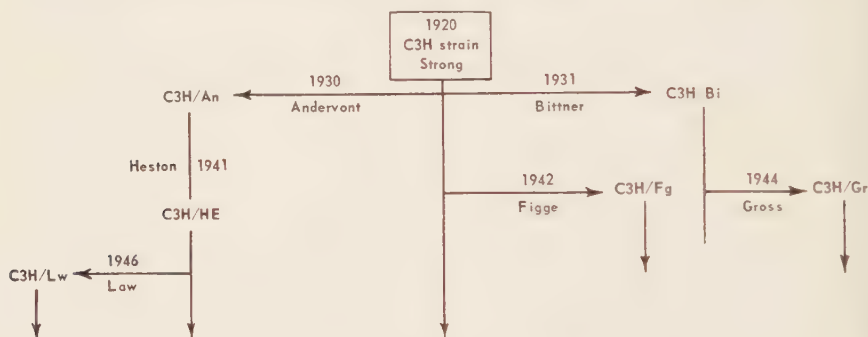


FIGURE 1. Genealogical diagram of certain sublines of the C3H strain of mice.

Of particular interest are the 2 sublines C3H Fg and C3H Bi. The Fg subline will be discussed now, and reference to the Bi subline will be made later. In 1952 Figge⁵⁹ first noticed the appearance of what he called lymphatic leukemia in his colony of this subline, obtained from Strong in 1942 and maintained by sib matings. In 1953 we obtained litter mates of the F96 generation from Figge, from which a colony was started. A high incidence of neoplasms of reticular tissue was observed. In order to increase the life span of the mice of this subline, a litter was removed by Caesarian section and was fostered immediately on C3Hf Lw mice, without the mammary tumor milk agent. Thus, a breast-carcinoma-free subline, C3Hf Fg, was established from the mating ♀ 18706 × ♂ 18707. It may be seen from FIGURE 2 and TABLE 4 that this subline now was indeed high-leukemic. There was a galaxy of different morphologic forms of reticular neoplasms with 51 per cent lymphocytic neoplasms. Few of these latter were thymic in origin and, in contrast to AKR and C58, thymectomy did not influence the incidence or age at death from these neoplasms. Filtrates prepared from 12 spontaneous and 3 transplantable neoplasms have been completely negative in C3Hf Bi (Z) test mice employed in other laboratories as sus-

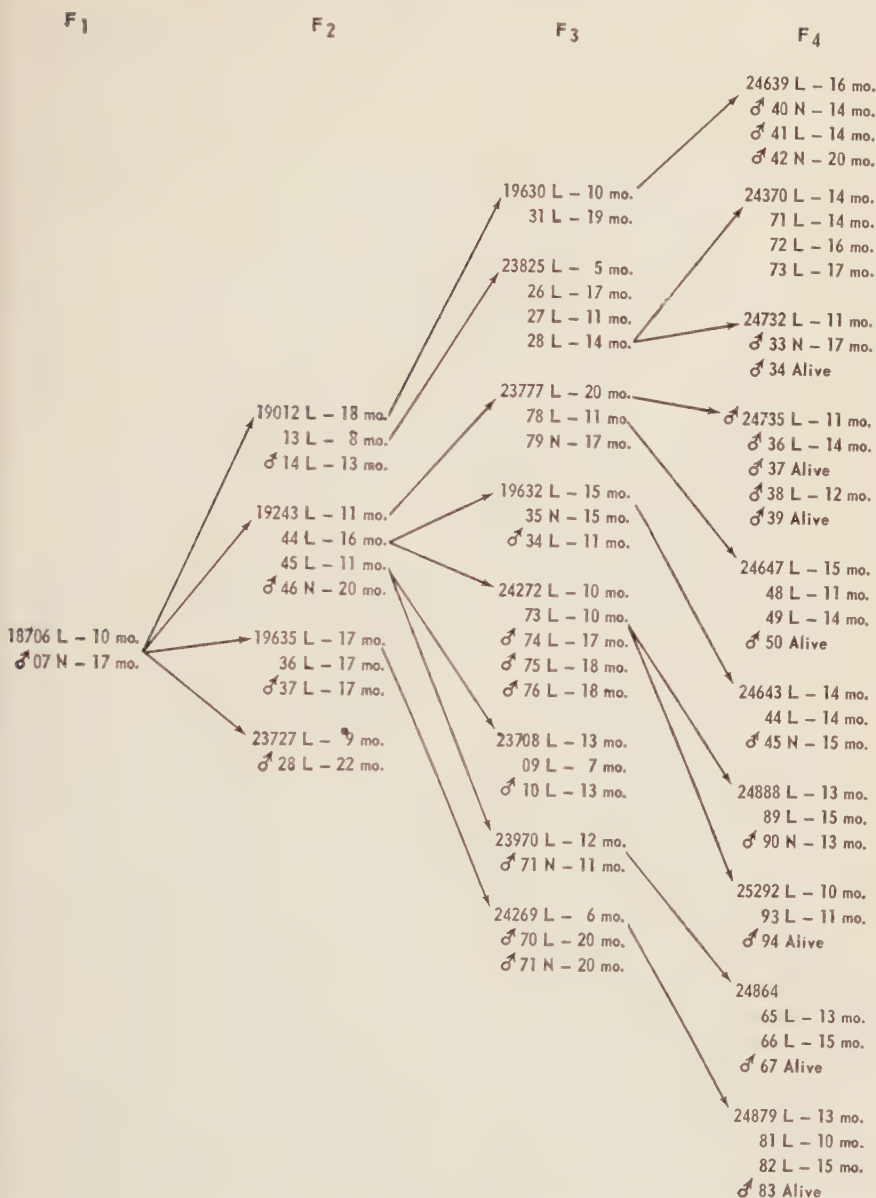


FIGURE 2. Pedigree chart through the first four generations of the C3H-Fg subline of the C3H strain. The numbers ♀ 18706 and ♂ 18707 represent the F₉₉ inbred generation. Since these mice were taken by Caesarian section and were foster-nursed on a milk agent-free subline, they are here designated as F₁. L = leukemia (reticular neoplasm); N = non-leukemic at autopsy; and A = still alive at the times given.

TABLE 4
RETICULAR NEOPLASMS IN THE C3H Fg SUBLINE OF MICE (F₁-F₅ GENERATIONS)

Sex	No. of mice	Leukemia		Mean age at death mo. (range)	Percentage of different morphologic forms*				
		No.	Percent-age		Ly	Type A	Type B	GR	PC
Females.....	73	59	80.9	13.2 (5-22)	51	6	17	11	13
Males.....	45	18	40.0						
Combined sexes.....									

* Ly = lymphocytic neoplasms; Types A and B reticular neoplasms; GR = granulocytic leukemia; PC = plasma cell neoplasms.¹

ceptible to certain cell-free filtrates. A progeny test is now in progress to determine the probable genetic basis of this high incidence of reticular neoplasms in this subline.

Of interest also has been the occurrence of 6 pleomorphic parotid gland neoplasms in approximately 200 untreated C3H Fg and C3Hf Fg mice in this laboratory.

Cell-Free Materials and Leukemia in Mice

In 1951 it was reported⁶⁰ that mice of the C3H strain developed leukemia if inoculated when less than 24 hours of age with extracts prepared from leukemic tissues or from tissues of embryos of the high-leukemic AK strain. Objections were made at this time to the interpretation of these observations, chiefly because of the use of extracts and centrifugates rather than filtrates, but also because of the employment of AK and C3H strains that share the same histocompatibility gene (H-2^k), and on the basis of other considerations.^{61, 62} No morphologic characterization of the leukemias arising in the C3H strain was given either at that time or later.

It was emphasized^{57, 60} that the C3H strain was nonleukemic. This, then, would appear to represent induction rather than enhancement of leukemia in the particular sublines of the C3H strain that were used.

Stewart,⁶³ but not Law *et al.*,⁶⁴ found an increase in the incidence of lymphomas in F₁ hybrid mice (C3H × AKR) following the introduction of filtrates and centrifugates prepared from AKR and other high-leukemic tissues. In our laboratory this F₁ hybrid normally has an incidence of lymphomas of 50 to 60 per cent, occurring as early as 7 months of age. An earlier appearance of these neoplasms results also from the use of fractionated radiation¹² or from the introduction of AKR (but not C3H) bone marrow.¹² Thus, enhancement of reticular neoplasms in this F₁ hybrid may be obtained by several methods.

Later it was reported⁶⁵ that newborn C3H mice of the Bittner subline were substantially more susceptible to the leukemogenic action of the so-called

"AK leukemic agent" than were those of an NCI subline (the An line). Distinctions between the two sublines employed, C3H/An and C3H/Bi (FIGURE 1), were not made in the earlier publications.

Our continued investigations in this field were therefore done with a subline of mice (Z or C3Hf/Bi) obtained from J. J. Bittner in 1954. Five families (I through V) of this subline were started from pedigreed litters. In addition, a small colony was derived from a litter of mice originally obtained from Bittner and kindly supplied by L. Gross (C3Hf/Gr).

In brief, the results to date employing the C3Hf/Bi subline as recipients of filtrates of spontaneous and transplantable AKR lymphocytic neoplasms are given in TABLE 5. Filtrates were injected when the mice were less than 12 hours old; identical filtrates were given to mice of the C3Hf/Lw subline

TABLE 5
RETICULAR AND PAROTID GLAND NEOPLASMS IN SUBLINES C3Hf/Bi AND C3Hf/Lw OF THE C3H STRAIN

Strain	No. of mice	Leukemia		Parotid gland neoplasms	
		No.	Percentage	No.	Percentage
C3Hf/Bi { (a) Experimental	(387) 159	10	6.9	5	3.2
(b) Controls	63	3	4.8		
C3Hf/Lw	(102) 86		None		None

Leukemia in (a) at 3, 4, 4¹/₂, 5, 5, 7, 9, 9, 9, and 10 months; leukemia in (b) at 11, 13, and 14 months.⁶⁴

shown previously not to respond as far as the induction of lymphomas was concerned. Following injection of the filtrates, profound differences in mortality were noted in these 2 strains; more than 60 per cent of the *Bi* subline died during the first week, in contrast to approximately 15 per cent of the *Lw* subline.

It is clear that some lymphocytic neoplasms made an early appearance in these test mice following injections of leukemic filtrates. It is also clear that the C3Hf/Bi subline in our laboratory was not a nonleukemic line. By 14 months there were 3 (4.8 per cent) lymphomas among the litter-mate controls. In contrast, however, these appeared later and were not lymphocytic neoplasms (there were 2 type A reticulum neoplasms and 1 undifferentiated plasma cell neoplasm). Two of the neoplasms in the experimental group, at 9 and 10 months, were type A and type B reticular neoplasms; the others were generalized lymphocytic neoplasms.

Additional information on the incidence and morphologic pattern of leukemia has been obtained from our breeding colony of C3Hf/Bi mice. TABLE 6 shows results to date in Family III, constituting the F78 to F81 inbred generations. Many of these lymphomas were lymphocytic neoplasms similar to those observed in the C58 and AKR strains. They grew pro-

gressively and, upon transplantation, killed only C3Hf/Bi strain mice. The picture was similar for the other families under observation, and for the smaller nucleus of C3Hf/Gr mice. Details on the incidence and morphology of these neoplasms will be published later. The results with Family III are shown here and are of interest because of the appearance of spontaneous pleomorphic parotid gland neoplasms (see also the description of neoplasms in the C3Hf/Fg subline). The incidence of lymphomas in the C3Hf/Bi subline is difficult to assess at present, since many mice are still alive. Further, the abilities to develop lymphomas may be far greater than expressed in the incidence of the disease, since many mice die at 20 to 24 months with severe amyloidosis.

TABLE 6
SPONTANEOUS LEUKEMIA AND PAROTID GLAND NEOPLASMS IN FAMILY III, C3Hf/Bi(Z)
SUBLINE OF THE C3H STRAIN

F78	F79-81
♀ 222512*—L—14 mo.	20 litters—64 mice
222513	6-Leukemias
222514	(11, 14, 16, 16, 16, and 19 mo.)
222515	5-Parotid gland neoplasms
222516—L—18 mo.	(4½, 6, 6, 6, and 6 mo.)
♂ 222511	

* The original pedigreed litter was obtained from Bittner; these, along with the off-spring constituting F79 to F81 inbred generations, were bred and observed in our laboratory. ♀ 222512 and 516 were leukemic at 14 and 18 months, respectively.

A smaller number of C3Hf/Bi mice received centrifugates (9500 rpm) rather than filtrates, prepared from AKR leukemic tissues. Several lymphocytic neoplasms were observed in this group. Two, one appearing at 11 months and one at 9 months, grew progressively only in AKR mice and not in C3Hf/Bi mice (see also Furth *et al.*⁶⁶). Four of 10 lymphocytic neoplasms in the filtrate group (TABLE 5) were transplanted to AKR and C3Hf/Bi mice. These, in contrast, grew progressively and were maintained in C3Hf/Bi mice.

It is clear from our preliminary observations with the C3Hf/Bi subline mice that (1) this subline is not nonleukemic in our laboratory; therefore one must distinguish between induced* (enhanced?) and spontaneous neoplasms; (2) the evidence presented by Gross on "vertical transmission

* By induction is meant the appearance of new neoplasms or neoplasms not regularly observed following some experimental procedure. Obviously, stringent control practice must be followed in order to establish that one is dealing with induction. By enhancement is meant a decrease in the latent period (earlier age at death) and/or an increase in the incidence of neoplasms that occur normally. In DBA/2 mice, for example, 30 per cent or more reticular neoplasms were observed in older mice of this subline. Reticular neoplasms, but principally lymphocytic leukemia, following X-ray or carcinogen exposure were observed to occur at an earlier age and in a larger number.

of the so-called leukemia virus must be viewed with caution*; and (3) the probability of transmitting leukemic cells exists if centrifugated extracts are used as the inoculum. This is borne out by the transplantation patterns of the neoplasms arising in mice receiving centrifugates (see also Furth *et al.*⁶⁶).

Discussion and Summary

Many different morphologic forms of neoplasms of the reticuloendothelial, lymphatic, and hematopoietic systems of the mouse have been observed and described. In addition, disturbances of these systems that give the appearance of neoplasms have been encountered. The investigator should make some attempt to determine whether he is dealing with a neoplasm, and he should also try to characterize the morphologic form of the neoplasm, whether lymphocytic, granulocytic, monocytic, plasma cell neoplasm, or the like. In most cases it is particularly useful to transform the neoplasms into an ascitic form.

The common form encountered in the mouse is the lymphocytic neoplasm; this expresses itself usually as lymphocytic leukemia.

Carcinogenic hydrocarbons, estrogens, and irradiation evoke lymphocytic neoplasms in mice. The role of these compounds in evoking type A and B reticular neoplasms, plasma cell neoplasms, and others has not been assessed fully.

Strain differences exist in the response to these different leukemogenic agents. It appears that enhancement of these neoplasms occurs in some inbred strains of mice while, in others, the neoplasms appear to be induced; that is, they are new neoplasms and are not ordinarily encountered spontaneously within the strain. Limited evidence indicates that the incidence of induced lymphomas is proportional to the total dosage, and that synergism between two of these agents is possible in certain experimental situations.

One need not always implicate an exogenous leukemogenic agent. Profound effects resulting from gene abnormalities are known, and in all probability result in the so-called "molecular diseases." Gene-controlled synthesis of abnormal hemoglobin molecules leads to sickle cell anemia.⁶⁷ Manifestations of the disease, phenylketonuria, result from the lack of a specific enzyme involved in the conversion of phenylalanine to tyrosine.⁶⁸ This disease is gene-controlled. High-leukemic strains such as AKR, C58, F, and C3Hf/Eg, develop neoplasms spontaneously. Susceptibility has a genetic basis that resembles the inheritance of susceptibilities to mammary tumors and lung adenomas.

In most (but not all) high-leukemic strains of mice the presence of thymic tissue appears to be necessary for the expression of lymphocytic neoplasms. Such is also the case in X ray-induced and carcinogen-induced leukemia in certain strains of mice. Isologous or autologous grafting of thymic tissue into a subcutaneous site restores the ability to develop leukemia (lympho-

* Only cell suspensions and centrifugated extracts were used in the experiments designed to show vertical transmission of a "leukemia agent." The mean age of C3H mice (undesignated sublines) dying from leukemia was 15.5 months, with a range from 8.5 to 20 months. Only first-generation offspring from inoculated parents were described in this report.⁶⁹

cytic). Through the use of compatible but genetically different thymic graft and host and, particularly, in thymectomized, irradiated animals, the following interesting facts have emerged:

(1) The existence of an indirect mechanism of induction of lymphocytic neoplasms is operative. Thymic tissue grafted as late as 28 days after the last X-ray exposure becomes leukemic.

(2) Grafted thymic tissue may be considered a "sphere of influence" responsible for the induction of lymphocytic neoplasms. In many instances host cells of the lymphoid series that have repopulated the thymic graft become leukemic. Further study is needed on the relationship between two phenomena: (1) the lymphocytosis-stimulating substance of certain neoplasms including, particularly, thymic tissue,^{6,16, 6,16} and (2) the lymphopoietic hyperplastic alterations in regional lymph nodes resulting from extracts and implants of thymic tissue⁷⁰ (both influences probably are related directly to epithelial components of the thymus). The induction of lymphocytic neoplasms in nonthymic tissue also needs further study.

(3) Within certain time limits, "conditioning" of the host following fractionated irradiation is reversible by intact antologous, but not homologous, bone marrow cells.^{71, 72}

(4) Genetic susceptibility to the spontaneous development of lymphocytic leukemia, or in X ray-induced neoplasms, is manifested as an intrinsic property of the thymic tissue itself. Thymuses from low-leukemic strains, or from strains refractory to X rays, do not become leukemic or exhibit the sphere of influence.

The role of cell-free materials in the spontaneous occurrence of leukemia in mice and in the X ray-, estrogen-, and carcinogen-induced diseases is by no means clear. It has been stated that filtrates from tissues of high-leukemic mice induce leukemia in a nonleukemic C3H Bi strain. It appears that such materials may merely enhance the appearance of these neoplasms in a manner similar to that observed in (C3H \times AKR)F₁ mice and in the AKR strain.

Although several investigators accept the viral nature of the filterable agent(s) in mouse leukemia, it is clear that no one has described properties specific to this agent that would set it apart from other possible biochemical complexes that possess leukemogenic activity.

Evidence for the "vertical transmission" of the "agent,"⁶⁰ that is, from parents to offspring, comprises only two generations. Filtrates were not used, and the mean age at death from leukemia is certainly within the range of spontaneously occurring lymphomas in the C3Hf Bi subline (at least as observed in our laboratory). On the other hand, preliminary data do suggest that genetic continuity exists, as shown by the apparent self-perpetuating characteristic seen in serial passage.⁷³

If leukemia in high-leukemic strains such as AKR and C58 is an "egg-borne" disease, as suggested, it is clear that male mice transmit the disease as well as females do, as demonstrated by identical expression in reciprocal F₁ hybrids. This would indicate that the spermatozoan is the vehicle of transfer, since it is known that mother's milk is ineffective and that, unlike

the "milk agent," transfer across the placental barrier apparently does not occur.

The observations of Graffi *et al.*,⁷⁴⁻⁷⁵ Schmidt,⁷⁶ and Friend⁷⁷ would appear not to be pertinent to the present discussion. The filterable agents concerned were obtained from Ehrlich carcinomas or other mesenchymal or epithelial tumors, some closely related to the Ehrlich. It must be established by these investigators that the diseases evoked by cell-free filtrates were neoplastic. It is stated, for example, that the Agnes-Bluhm strain used by Graffi and Schmidt was highly inbred, yet it was reported that, upon transplantation into other members of this strain, only a few of the lesions grew progressively.

The origin of high-leukemic lines is of special interest. This phenomenon has been explained, after the fact, by the demonstration of a genetic basis for the expression of the disease (in the AK and C58 inbred strains). The pattern of inheritance is similar to that shown for breast cancer in mice, the expression of which in certain situations depends in part on the presence of a viruslike agent. Expression of breast cancer in other situations is not dependent upon the presence of the milk agent. Unlike breast cancer, however, it has been impossible to establish or eliminate leukemia from inbred lines by known biological techniques.

The end point of expression of reticular neoplasms in the C58 and AKR strains has been the emergence of a common morphologic form, the lymphocytic neoplasm, chiefly thymic in origin. Data now exist to show a new high-leukemic subline, C3H₁ Fg which, unlike AKR and C58, has a background of diverse neoplastic cell types.

If gene mutations, necessarily of recent origin, are in part responsible for the expression of the disease in this subline, the "progeny test," as described earlier, should reveal this.

The claim of the establishment of a "leukemic agent" on C3H genetic soil ("vertical transmission") has been used to demonstrate the likely possibility that leukemia in the mouse is an "egg-borne" disease similar to lymphomatosis in chickens. This discrepancy of claim and fact should be resolved at the earliest possible moment.

It is clear that many variables are concerned with the expression of reticular neoplasms in the mouse. Some information is available relating to the mechanisms involved in the evocation of neoplasms by certain factors, and the interrelationships among certain neoplasms are being established, particularly with respect to the extent to which they influence the expression of lymphocytic neoplasms.

The relationship of certain acellular materials to the spontaneous disease, or to its induction or enhancement by X rays, estrogens, and carcinogenic hydrocarbons, has not been established as yet. It would indeed be premature, on the basis of present evidence, to state that "mouse leukemia was demonstrably caused by a filterable virus," that the disease is an "egg-borne virus disease," or that "the mouse leukemia virus is a spherical particle less than 70 m μ in diameter." The burden of proof rests with investigators who make these claims.

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RECENT STUDIES ON THE MOUSE MAMMARY TUMOR AGENT*

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The viruslike characteristics of the mammary tumor agent (MTA) have been discussed in many review articles.¹⁻¹² Not long after it was shown that the agent was normally transferred in the mother's milk¹³ and that other causative factors must interact in the genesis of mammary cancer in mice,^{14, 15} it was demonstrated that the MTA could be administered in extracts of either normal or cancerous tissues from infected mice,^{2, 16-20} including seminal vesicles²¹ and cauda epididymidis.²²

During the past ten years various workers have found that, in certain matings, when agent-free females of some inbred strains, either susceptible or relatively resistant to the development of spontaneous mammary cancer, were mated with males of cancerous strains, a relatively high incidence of mammary cancer might be observed in the progeny, especially in those born in the later litters. It has been accepted that the development of these tumors was dependent upon the transfer of the agent by the male, first infecting the mother, who then transferred the MTA to her offspring in her milk.^{8, 9, 20-33}

This discussion will be concerned primarily with observations on male transmission of the MTA, as determined by the incidence of mammary cancer seen in the females and their progeny, correlated in some instances with biological assay of the tumors for the MTA.

Materials and Methods

The material discussed will be limited to only a few inbred strains and their hybrids.

Animals of the cancerous Andervont subline^{1, 21, 23-26, 34-37} of the C3H stock, originally developed by Strong,^{38, 39} were obtained in 1948. Although some were fostered later, it appeared likely that they possessed the agent, as no significant difference was noted in the development of mammary cancer in their hybrid progeny.²⁹ Our C3H line was separated from Strong's stock in 1931; to simplify the designation of hybrids, those of the cancerous line have been called the Z, or Z(C3H), subline, while those of the fostered agent-free group are known as the Zb line;^{27, 29} mice of the Andervont substrain will be called C3H/AnBi animals.

Another group of Z mice nursed a lactating female of the A stock and obtained the MTA of that cancerous strain. The individuals of the fostered

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litter were called Za1; of the next generation, Za2; the data given in the tables are for mice of four generations (Za1 to Za4). Should it be necessary to compare animals of the Z(C3H) line with the Za mice, Zz or Z mice with the z agent will again be used to describe the former.^{7, 29}

Representatives of the Bagg albino stock were secured from Snell in 1942, and the letter "C" will be used^{7, 28-30} instead of "BALB c." Five male mice, the progeny of C mothers injected with an extract of a transplanted mammary cancer, were also employed. This tumor had developed spontaneously in a female of the cancerous A stock and was carried for one passage in agent-free animals of the same strain.

Usually five C females were mated with males of the various stocks, and some of their progeny were observed for the development of mammary cancer. In certain groups, details will be given concerning the breeding program, in that they were permitted to bear only a definite number of litters by one male before another was substituted. Other C females had their uterine horns amputated at the junction with the body of the uterus, with the ovaries *in situ* and intact.³⁰ Although these females were unable to have progeny, they were placed in breeding pens with males of the cancerous Z(C3H) stock together with unoperated litter-mate controls, using two females of each group per pen.

Preliminary data, obtained with the collaboration of M. J. Frantz, may be cited where C females were housed with vasectomized Z males for 155 to 219 days, after which the C females were mated with intact, agent-free males of the C, Zb, or I³⁹ stock. The Z males were operated on when they averaged 5 weeks of age and, soon after the operation, were "mated" with C females of weaning age.

In designating the hybrids, the maternal parent was always given first; for example, C ♀ × Z(C3H) ♂ = CZF₁; C ♀ × Zb ♂ = CZbF₁; or C × C3H AnBiF₁. Several mammary tumors were assayed for the MTA by injecting extracts of the tumors into agent-free ZBC mice. The method of preparing the cell-free centrifugates for administration was similar to that previously described,^{7, 8, 28, 29, 40, 41} and the ZBC animals were from 23 to 30 days of age. Litter-mate controls were employed in most experiments.

Results and Discussion

Observations on the development of spontaneous mammary cancer in breeding females of the various inbred strains are tabulated in TABLE 1, where the average ages (development of cancer or death of noncancerous mice) are expressed in days. In excess of 90 per cent of the C3H breeders died with mammary cancer, and the primary difference between mice of the various groups was noted in the average ages at the time when their tumors appeared. In the Z(C3H) mice with the MTA secured from the cancerous A strain, the average cancer age for the entire group was approximately two months later than was observed in the unfostered Z animals; the age in the Za females became later with each succeeding generation.²⁹

The four mothers of the C strain that received the MTA by the administration of an extract of a transplanted mammary tumor had tumors at an

TABLE 1
OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER IN BREEDING FEMALES OF
SEVERAL INBRED STRAINS

Stock	Number of mice	Cancerous (per cent)	Average age in days	
			Cancerous	Noncancerous
Z(C3H).....	599	97	272	272
C3H/AnBi.....	136	91	228	227
Za.....	80	93	321	397
Zb.....	700	0.4	482	475
A.....	352	90	302	
C.....	235	0.4	535	589
C*.....	50	74	338	627

* See text: progeny of C females that obtained the MTA by administration of extract of the tumor (A stock).

TABLE 2
DEVELOPMENT OF MAMMARY CANCER IN FEMALES OF THE C STOCK AFTER BEING MATED
WITH MALES OF VARIOUS STOCKS, ESPECIALLY THE CANCEROUS Z(C3H) SUBLINE

				Average age in days		Average No. litters		
				Cancer- ous (per cent)	Cancer- ous	Noncan- cerous	Cancer- ous	Noncan- cerous
	No.							
C ♀ mated Z(C3H) ♂	1947....	8	50	544	686	9.0	10.3	
C ♀ mated Z(C3H) ♂	1949....	14	63	483	688	9.9	9.6	
C ♀ mated Z(C3H) ♂	1950....	5	60	558	533	8.0	7.5	
Controls for C ♀	1951....	14	50	495	720	8.0	6.5	
with amputated uterine horns	1951....	17	53	564	666	operated ♀ ♀		
Mated Z ♂ for 5 litters, then Zb ♂	1953....	5	60	545	565	7.7	6.0	
Total.....		63	56	522	659			
Mated Z ♂ for 4 litters, then Zb ♂ (2 groups).....	19	16	576	579	7.0	7.0		
Alternate matings: Z & Zb ♂ (2 groups).....	17	0	—	639	—	6.7		
× vasectomized Z ♂ (155-219 days) then Zb, C, I ♂.....	18	17	403	663	3.1	5.7		
× Za ♂ (MTA of A stock).....	9	22	482	646	8.5	9.0		
× Ca ♂ (prog. of injected C ♀)...	25	24	510	669	7.5	7.5		
× C3H/AnBi ♂.....	20	10	599	621	7.5	6.9		
× Zb ♂ (agent-free).....	14	0	—	671	—	8.4		

average age of 423 days, and a high incidence was observed in their female progeny (TABLE 1). Five male offspring were studied for the transmission of the agent.

Only C females used to maintain the inbred line were tabulated; these were continued for longer than 450 days. One mammary tumor, an adenocarcinoma with considerable squamous metaplasia, was observed among this group (TABLE 1). Other C females, numbering 174, were observed for more than 300, but not to 450 days, and all died noncancerous.

During the past several years 6 groups of C females have been mated with males of the cancerous Z(C3H) stock. Of these 63 females, 35 (56 per cent) developed mammary cancer at an average age of 522 days. The various groups consisted of 5 to 17 animals; the lowest incidence to be observed in any series was 50 per cent, the highest 63 per cent, regardless of the number of mice. Included were 17 females with amputated uterine horns, which showed an incidence similar to that of their intact litter-mate controls (TABLE 2).

TABLE 3 shows data on the development of mammary cancer among the offspring born to a few C mothers after they were mated with males of the cancerous Z(C3H) or C3H AnBi sublines. The progeny are recorded by litters; the number of either cancerous or noncancerous animals is followed by the average age of each. One of the C females, No. 154980, developed mammary cancer when it was 662 days of age, after having 9 litters. The

TABLE 3
DEVELOPMENT OF MAMMARY CANCER IN THE HYBRID PROGENY OBTAINED BY MATING
C FEMALES WITH CANCEROUS Z(C3H) OR C3H/AnBi MALES

Litter	C ♀ 154980 + 662(9) × Z(C3H) ♂		C ♀ 154984 - 663(9) × Z(C3H) ♂		C ♀ 154987 - 402(7) × Z(C3H) ♂		C ♀ 168865 - 687(7) × C3H/AnBi ♂	
	Cancer- ous	Noncan- cerous	Cancer- ous	Noncan- cerous	Cancer- ous	Noncan- cerous	Cancer- ous	Noncan- cerous
1st	0	2 = 764	0	0	0	2 = 519	3 = 564	0
2nd	0	3 = 699	1 = 557	2 = 742	1 = 436	4 = 667	0	1 = 383
3rd	3 = 204	0	1 = 511	2 = 710	0	0	2 = 584†	1 = 658
4th	2 = 204	1 = 272	1 = 649	4 = 714	3 = 197	0	0	0
5th	3 = 238	0	0	0	5 = 241	0	0	0
6th	4 = 185*	0	0	5 = 695	4 = 273	0	1 = 789	2 = 834
7th	0	0	0	3 = 675	0	0	0	0
8th	5 = 262	0	3 = 213†	1 = 721				

The age of the mothers is denoted by + (cancer) or - (death); the number of litters are shown in parenthesis. The number of progeny and the average ages are expressed as: No. = average, by litters.

* No. 8957 (TABLE 4, Exp. 7: assay data).

† No. 9009 (TABLE 4, Exp. 8: assay data).

‡ No. 9467 (TABLE 9, at 639: assay data).

5 CZF₁ hybrids born in her first and second litters died noncancerous but, starting with the third litter, 17 of 18 of the young born in the succeeding litters had mammary cancer at early average ages. One tumor from a female of the sixth litter was tested and found to have the MTA (TABLE 4, EXPERIMENT 7). Another C female, No. 154984, cast 9 litters, yet died noncancerous when 663 days of age. Of her 19 progeny born in her first

TABLE 4

ASSAY FOR THE MTA OF TUMORS FROM C FEMALES AND THEIR PROGENY, COUNTING ONLY NONCANCEROUS ANIMALS THAT LIVED TO THE AVERAGE CANCER AGE

Experi- ment No.	Details regarding tumor	Gram equivalent injected		Gram equivalent injected			
		10 ⁻¹ *	2 × 10 ⁻⁴ *	10 ⁻² **	10 ⁻³ or 10 ⁻⁴ *		
		No.	Cancer- ous (per cent)	Age	No.	Cancer- ous (per cent)	Age
1	C ♀ + 500 (× Z ♂, 8 litters)	28	71	308	31	84	342
2	C ♀ + 495 (× Z ♂, 9 litters)	30	77	319	51	76	347
3	C ♀ + 610 (× A ♂, 11 litters)	27	70	441	29	76	493
4	C ♀ + 265 (vas. Z ♂, 194 days)	24	54	359	24	67	331
				17% liv.			17% liv.
5	CZF ₁ + 127 (mother + Exp. 2)	27*	85	295	{ 29 39*	96 95	313 324
6	Vg. CZF ₁ + 432 (C ♀ = -658, 11 litters)	35	80	399	39	79	347
7	CZF ₁ + 185 (C ♀ = +662, 9 litters)	32	72	396	40	88	347
8	CZF ₁ + 179 (born 8th litter to C ♀ 663, 9 litters)	16*	81	354	{ 35 21*	84 71	316 310
9	CZa ₃ F ₁ + 301 (C ♀ - 623, 10 litters)	11	73	355	15	100	323
10	CZbF ₁ + 556	19	0	—	23	4	465

The asterisks indicate the dilutions injected, as cited above each column.

7 litters, 3 had tumors at an average age of 572 days. These cancerous animals were members of the second to the fourth litters, yet 8 of the sixth and seventh litters died noncancerous. In the eighth litter, 3 of 4 died cancerous at an average age of 213 days, and 1 tumor was shown to possess the MTA; the extract containing 10⁻¹ gm. equivalents of material was as active as when the extract was diluted only one-hundredfold (TABLE 4, EXPERIMENT 8). Another female, No. 154987, was found to be infected with the agent from the Z male by the time her fourth litter was born; possibly she would have developed cancer if she had lived beyond 402 days.

The pedigree of C female, No. 168865, and her progeny produced by the cross with a male of the cancerous C3H AnBi subline was quite different than when Z males were used (TABLE 3). This will be considered in detail later.

As may be seen from the data presented in TABLE 3, the development of mammary cancer occurred in the progeny of all infected C females mated with Z males, whether the mothers did or did not give rise to the disease. The results for all matings have been tabulated in TABLE 5. Whereas only a small proportion of the CZF₁ hybrids born to noninfected C mothers had cancer, tumor developed in nearly all (96 per cent) of those cast by infected C females, and this development took place at an average age earlier than was seen in breeders of the paternal Z stock (TABLE 1). Before these same C females became infected, however, the observations recorded for their progeny were similar to the offspring born to mothers that remained noncancerous and noninfected. Sixty-three cancerous CZF₁ females were members of the first litters after their C mothers became infected; they had an average age of 245 days, almost identical with the average age for the entire group born to the same mothers (TABLE 5).

TABLE 5
OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER IN THE CZF₁ PROGENY,
DEPENDING UPON WHETHER OR NOT THE C MOTHERS BECAME INFECTED WITH THE
MTA FROM THE Z MALES

C mothers	CZF ₁ progeny	Cancerous (per cent)	Average age in days	
			Cancerous	Noncancerous
Noncancerous, noninfected.....	128	5	633	723
Cancerous, before infection.....	69	6	585	722
Noncancerous, before infection.....	101	6	565	691
After infected—C mothers died, cancerous or noncancerous.....	141	96	242	369

These data would indicate that the tumor-producing properties of the MTA transferred by the C mothers to their first litters following infection were as active as the agent transferred to mice of the last litters; in some instances these might be either the third or eighth litters, respectively. Similar results have been reported^{40, 41} concerning the development of mammary cancer in the progeny of mothers that obtained the MTA by the administration of extracts of different dilutions (unpublished data).

In previous experiments it has been determined that the C females might become infected with the agent any time between the third and eighth litters.²⁸⁻³⁰ Recently, tumors from members of the first and second litters have been tested, but sufficient time has not elapsed since the administration of the extracts to know if the experimental animals may develop tumors due to the MTA.

As may be seen from the data given in TABLE 2, the percentage of C females to become infected with the agent, including the later development of mammary cancer, was influenced by the number of litters sired by males of the cancerous Z stock. Five C females were mated with a Z male for 5 litters and then with an agent-free Zb male: 3 died with mammary cancer (TABLE 2), another infected female died noncancerous, while the fifth remained non-infected. Only preliminary data may be given for the progeny, but in litters born after the 4 C females became infected, their 15 CZF₁ progeny all had cancer at an average age of 254 days. Four hybrids that had Zb fathers were observed; of these four, 3 had cancer at an average age of 205 days. One CZbF₁ tumor was assayed, and tumors are now appearing in the test animals.

Nineteen other C females were mated with Z males for only 4 litters, and then Zb males were substituted (TABLE 2). Three of the C females developed mammary cancer, an incidence of 16 per cent. Fourteen of the noncancerous females lived for longer than 500 days, and 18 of the total had progeny that were observed from the fifth and/or succeeding litters. Four C females were infected, including 2 noncancerous females and, of their 17 CZbF₁ progeny, 16 developed mammary cancer at an average age of 268 days. Preliminary data indicate that the 1 tumor from a C female that was tested possessed the agent, as did 1 of 3 CZbF₁ tumors; the other experimental mice were so young that tumors were not expected to develop in them.

The third C female, No. 183448, developed cancer when 681 days of age, after having had 10 litters, the last 6 sired by a Zb male. The tumor was diagnosed as an adenocarcinoma with squamous metaplasia and, in addition, the female had an adenoma of the lung. A total of 12 progeny were observed, 5 CZbF₁ hybrids of the third and fourth litters, and 7 CZbF₁ animals of the fifth to the eighth litters. Two, 1 in each group, had mammary cancer at an average age of 396 days (the tumor from the CZbF₁ female was an acanthoma). The 10 noncancerous hybrids survived to an average of 680 days, and only 1 had less than 4 litters. Although none of the tumors was tested, the low incidence in the progeny, the age at which the C female developed cancer, and the histology of the tumor would indicate that the female had not become infected with the agent from the Z male.

Twenty-four C females were placed in pens with vasectomized Z(C3H) males and, after remaining for 5 to 7 months, were mated with intact agent-free males of the Zb, C, or I⁺ stocks. Only those that had young were tabulated (TABLE 2); of these 18, 3 developed mammary cancer. The details concerning these cancerous mice are as follows:

Female No. 197413 remained with a vasectomized Z male for 194 days, and was then mated with a male of the I⁺ stock. She had one litter, but failed to raise any progeny, and she developed mammary cancer when only 265 days of age. As demonstrated by biological assay, the tumor from this C female possessed the MTA (TABLE 6 and TABLE 4, EXPERIMENT 4). In this experiment the amount of the agent from the extract diluted one-thousand-fold showed a higher activity, both for incidence and cancer age, than did the extract from 2×10^{-2} gm. equivalent of tissue. Another cancerous C

TABLE 6
ASSAY OF A MAMMARY TUMOR THAT APPEARED AT 265 DAYS IN C ♀, No. 197413

Gram equivalent injected	No.	Cancerous (per cent)	Average age in days	
			Cancerous	Noncancerous
2×10^{-2}	24	54	359	460
10^{-3}	24	67	331	504

The female had been housed for 194 days with a vasectomized male of the cancerous Z(C3H) stock, and then mated with an agent-free I male. Preliminary data: 17 per cent living in each group.

female, No. 196689, was mated with a Zb male after being in the same pen with a vasectomized Z male for 155 days. Three litters were born, and the C mother had a tumor when she was 312 days of age. None of the first litter lived to weaning age, but 9 CZbF₁ progeny of the second and third litters all had mammary cancer at an average age of 244 days (TABLE 7). As tumors are developing in the injected animals, 1 tumor has been assayed and found to have the agent. The third C female, No. 197167, developed a tumor at the age of 631 days. She had been mated with a vasectomized Z male for nearly 6 months, and later had 6 litters sired by a C male. Five progeny from this mating were observed, but none developed cancer. The tumor from the C mother was not tested for the agent.

After 195 days with a vasectomized Z male, C female No. 194715 was mated with an I male. She had 4 litters and died noncancerous at the age of 452 days. Seven of her 12 C1F₁ offspring have had tumors at an average age of 203 days; 4 are living, and the other died without cancer at 368 days of age. One tumor that appeared at 216 days in a member of the fourth litter was tested for the agent but, after 7 months, tumors have not appeared in the injected mice.

When males of the cancerous Z stock secured the MTA of the cancerous A strain (obtained by foster nursing) and were mated with C females, only 2 of 9 had mammary cancer, although 5 others became infected with the agent from these Za males.²⁹⁻³⁰ The CZaF₁ progeny born to the infected C females

TABLE 7
INFORMATION ON THE DEVELOPMENT OF MAMMARY CANCER IN THE PROGENY OF C ♀
No. 196689, WHICH DIED CANCEROUS AT 312 DAYS OF AGE AFTER BEING HOUSED
WITH A VASECTOMIZED Z MALE AND LATER HAVING PROGENY BY A Zb MALE

C ♀ 196689 + 312: housed with vasectomized Z(C3H) ♂ until 155 days of age, then mated with Zb ♂ and cast 3 litters.

First litter: born when female was 201 days; all died before weaning.

Second litter: born when female was 238 days; 4 females continued, all died cancerous; average age = 236 days.

Third litter: born when female was 285 days; 5 females continued, all died cancerous; average age = 250 days.

TABLE 8
OBSERVATIONS ON THE DEVELOPMENT OF MAMMARY CANCER AMONG THE PROGENY OF
C FEMALES MATED WITH C MALES POSSESSING THE MTA

	Progeny	Cancerous (per cent)	Average age in days	
			Cancerous	Noncancerous
C Mothers:				
Eighteen noncancerous, noninfected.	158	4	527	603
Before infection:				
One noncancerous.....	5	20	585	647
Six cancerous.....	31	6	628	589
After infection:				
One noncancerous.....	5	80	298	325
Six cancerous.....	27	74	315	625

had an incidence similar to the $CZzF_1$ group, except that those of the former series developed their tumors at a later average cancer age.²⁹

In the present study, 5 C males, the progeny of C mothers injected with an extract containing the MTA from a transplanted tumor from the A stock, were mated with 25 C females, 6 of which had mammary cancer (TABLE 2); 1 became infected with the MTA, but died noncancerous, and the others remained noncancerous and probably were noninfected. The development of mammary cancer among their $CCaF_1$ progeny, depending upon the state of the mothers, has been presented in TABLE 8. Again, no difference was noted between the progeny born to the infected mothers, whether they did or did not have mammary cancer. The late average age was the primary difference between these and other hybrids born to infected mothers.²⁹

While Mühlbock²⁰ did not observe the development of mammary cancer in sterilized agent-free females after they had been "mated" with males of a cancerous stock, 53 per cent of our C females with amputated uterine horns eventually had the disease after being housed in breeding pens with males of the cancerous $Z(C3H)$ stock.³⁰ The incidence in intact litter-mate controls mated with the same group of Z males was 50 per cent (TABLE 2). Among the 14 control C females, 3 were infected with the agent. After infection, 98 per cent of the 44 CZF_1 progeny born to the infected C females had mammary cancer, at an average age of 239 days (included in TABLE 5). Only 3 per cent of the progeny had cancer when they were born to the same mothers before they became infected with the agent from the males, an incidence similar to the progeny born to noninfected, noncancerous C mothers, where 4 per cent of their 24 offspring had tumors.

Agent-free Zb males were mated with 14 C females, all of which died noncancerous (TABLE 2). The incidence among their $CZbF_1$ progeny, numbering 216 mice, was 5 per cent, with an average cancer age of 593 days.²⁹ One tumor was tested for the agent, and only 1 of 42 test animals died cancerous (TABLE 4, EXPERIMENT 10).

Two of 20 C females developed mammary cancer when they were mated

with males of the C3H/AnBi subline. Forty-two per cent of all hybrids ($C\eta \times C3H/AnBi\sigma$) had cancer at an average age of 609 days, but when only those noncancerous breeders that survived to 600 days (or to approximately the average cancer age) were counted, the incidence was increased to 51 per cent. Also, using the same criteria, the progeny born to noncancerous C mothers showed a higher incidence (52 per cent) than did the progeny born to the 2 cancerous C mothers, or 40 per cent (TABLE 5).²⁹ Other tabulations showed that 50 per cent of the hybrids born in the first 2 litters to all females developed mammary cancer, yet only 6 of the tumors appeared in females under 400 days of age.

Eight mammary tumors from C3H/AnBi σ F₁ hybrids were assayed for the MTA, but the biological tests failed to demonstrate any activity in the extracts of any tumor, regardless of the concentration of the administered material.²⁹ These data have been retabulated in TABLE 9, and compared with the preliminary results observed after 10 months of a recent experiment where another tumor from a hybrid of the same cross is being tested. As may be seen, 46 per cent of the 85 ZBC assay animals have already developed mammary cancer at an average age of 238 days, and only 1 has died without cancer. Again, the incidences in the test animals are not determined by the amount of the injected material. It seems probable that the agent from these tumors may be the most active to be tested at these dilutions in any experiment.

The C mother (No. 194198) of this $C \times C3H/AnBi$ F₁ hybrid had a cystadenocarcinoma of the mammary gland when she was 637 days of age, after having 7 litters. Seven progeny were continued from the third to the

TABLE 9
BIOLOGICAL ASSAY FOR THE MTA IN EXTRACTS OF SPONTANEOUS MAMMARY TUMORS FROM
HYBRIDS OF THE $C\eta \times C3H/AnBi\sigma$ CROSS

$C\eta \times C3H/AnBi\sigma F_1$	2×10^{-2} gram equivalent			10^{-3} gram equivalent		
	No.	Cancerous (per cent)	Cancer age	No.	Cancerous (per cent)	Cancer age
At 512 days.....	22	0	—	25	0	—
At 626 days.....	8	0	—	10	0	—
At 596 days.....	6	0	—	10	0	—
At 582 days.....	9	0	—	10	0	—
At 518 days.....	10	0	—	9	0	—
At 632 days.....	10	10	561	9	11	731
At 639 days.....	19	0	—	13	0	—
At 651 days.....	10	0	—	9	11	417
At 288 days:						
No. 9936.....	23*	35	250	19*	53	238
No. 9937.....	25*	48	247	18*	50	216
No. 9936 }.....	19*	26	250	19*	58	246
No. 9937 }						

* Preliminary data after 10 mo.

fifth litters. One had cancer at 637 days of age, 3 died noncancerous, and the others are still living. The female failed to raise any of the sixth litter, and 3 were continued from her seventh litter. All had mammary cancer at an average age of 230 days, and the tumor tested (TABLE 8) appeared, in the oldest, to have cancer at 288 days of age. As the agent could be recovered from a tumor of an offspring born in the seventh or last litter, it would appear likely that, had they been tested, the agent could have been demonstrated from the lactating mammary gland and/or the spontaneous mammary cancer of the C mother, although the latter did not become cancerous until she was more than 21 months of age. This interpretation would be consistent with the results obtained for other C females that died either cancerous or noncancerous and possessed the agent, either in their tumors or as shown by its presence in their progeny, after they had been mated with males of the cancerous Z subline.

As mentioned above, 8 tumors from $C \times C3H/AnBi$ F_1 hybrids were tested, but only 3 of the 189 experimental ZBC females were found to have cancer at an average age of 570 days. These observations compare with the incidence seen in noninjected ZBC breeding females.²⁸

Two of the tumors appeared in hybrids having the same C mother, one in the third litter and the other in the fourth litter, at an average of 604 days. These C mothers had 60 offspring (including noncancerous mice that survived to 600 days), and they showed an incidence of 53 per cent and an average age of 608 days, a showing that was almost identical with the observations recorded for the entire group of $C \times C3H/AnBi$ F_1 hybrids (51 per cent at 609 days).²⁹ Thirteen additional offspring died noncancerous between 400 and 600 days of age. When these are included, 67 per cent of the mice born in the same litters of which the assay animals were members had mammary cancer, at an average cancer age of 586 days. In the litters born to the C females in their preceding litters, 38 per cent died cancerous while, of the offspring born to the same C females in the litters following those containing the assay animals, 8 of 23 (or only 35 per cent) developed cancer at an average age of 670 days. The incidence for the total of 73 hybrids was 44 per cent, as in the entire group when noncancerous animals dying between 400 and 600 days are considered, but there was neither an increase in incidence nor an acceleration in the time of appearance of the disease in the hybrids born in succeeding litters to these C females. These observations, together with the almost complete absence of tumors in the experimental animals injected with extracts of the mammary tumors, would indicate that none of the C females had become infected with the MTA from the males of the C3H/AnBi subline, such as occurred in a majority of females in the $C \times Z(C3H)$ male cross. In one case where it was established that the C female became infected from the C3H/AnBi male, the C female died cancerous, the hybrid progeny born in the seventh litter all had cancer at an average age of 230 days, and tumors are appearing in a high proportion of the test animals and at an early average cancer age. The results are similar in hybrids of the 2 crosses in cases where the MTA may be demonstrated by biological assay.

The data reported for these investigations on male transmission of the

MTA would corroborate many other investigations. They also show that the results would be influenced by the strain of agent-free females, by the stock of the cancerous males, and by the origin of the MTA in the paternal line.^{1, 7, 9-11, 20-26, 28-34, 42-44} It has been demonstrated also that females with amputated uterine horns may become infected from the male, and that vasectomized males may transfer the MTA and infect agent-free females. In addition to the new information obtained concerning the etiology of mammary cancer in mice, these observations must also be considered, as well as any possible nursing influence, in any study made to ascertain whether the results secured on mice might explain some aspects of the human problem.⁸

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GENERAL SUMMARY

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To present an adequate general summary of this monograph would require the efforts of a virologist, a bacteriologist, a geneticist, an immunologist, and a pathologist. Since it is impossible for anyone to claim sufficient experience in all these disciplines, I shall speak as an investigator who has long been interested in the etiology of cancer. Before attempting this approach I should like to commend the contributors to this publication for the high quality of their presentations and to ask forgiveness when I fail to refer to their efforts and accomplishments. Such exclusion merely is indicative of my inability to grasp the full significance of their reports.

On behalf of those who participated in the conference on which this monograph is based, I express my appreciation to the co-chairmen, C. P. Rhoads and H. Koprowski, and to their able assistants, A. E. Moore and M. J. Kopac, for their originality in avoiding the discussion of a wide range of virus-induced tumors in plants and animals, and in proposing as topics, instead, three tumors in which viruses are clearly involved and one in which the role of a virus is receiving attention. These four neoplasms not only reveal the remarkable diversity of tumor viruses; they also enable us to evaluate some of the implications contained in the more general papers.

The first tumor I shall consider is fowl leukosis, since it was the first tumor proved to be of viral etiology. As studies progressed over a number of years, the problem, in common with most cancer research, became more complex. A variety of neoplasms that we now refer to as the fowl leukosis complex was exposed. We have heard how one form of this complex, visceral lymphomatosis, has yielded to intelligent, patient, and continuous investigation. It was found to be contagious as well as egg-borne and, while many chickens are infected, all do not develop the disease. For our purpose, however, the demonstration of a contagious tumor virus does much to dispel any air of mystery we may attach to cancer viruses.

In the community of cancer viruses this virus would rank as a "solid citizen" or, if we accept the suggestion that it is the stem virus of others in the complex, it could be called a "good father" type. It has successfully solved the problems of transmission and survival and, but for one mistake, would lead an unmolested existence: it kills some of its hosts, which makes it of economic importance to man. Any such virus must lead a precarious existence.

Two other highly active viruses of the complex have been established in the laboratory. They induce acute forms of the disease, and both have been studied diligently. We have heard how these investigations have revealed that the viruses of the complex possess similarities and dissimilarities and how, in all probability, they belong to a family of closely related strains.

This finding takes us back to the first paper of the monograph, which shows that a pure clone of virus can become contaminated by genetic variation. Perhaps the fowl leukosis viruses demonstrate that similar contamination confronts nature in the normal course of events. It likewise suggests that tumor viruses, and perhaps tumor cells, may also enjoy the privileges of transduction and transformation that have been so brilliantly described for bacterial cells.

Studies on the fowl leukosis complex suggest a relationship between these viruses and the virus of Rous sarcoma. This brings us to the papers dealing with the latter virus, which was the second prototype virus discussed in these pages.

The Rous sarcoma virus was discovered in 1911 and, after many years of animal passage, it has acquired a remarkably high degree of activity. It provokes tumors within a few days, and histological evidence of malignancy is present within hours following the administration of a relatively pure virus. Unlike fowl leukosis, it is not part of a complex; it initiates a distinctive tumor at the site of administration. Among cancer viruses it can be considered as the "classical cancer virus" because of its exceptional activity and the consistent tissue response it elicits in adult chickens.

Early response to this virus was tempting to investigators who wished to ascertain the sequence of events attending the transformation of normal cells to malignant ones. Such efforts require the use of purified virus, and we read of progress made toward purifying this virus. The undertaking has been difficult, but considerable progress has been made; this progress has shed light on virus problems in general. Of special interest to cancer research was the observation that a small amount of virus produced tumors from which the virus was not recoverable by the usual assay techniques. The implications here are clear. Inability to find viruses in tumors can be expected when the highly active Rous virus can be detected only with difficulty in tumors it has induced.

We have been presented with evidence showing that quantitative studies with this virus suggest that few—perhaps only one—virus particles can produce a tumor on the chorioallantoic membrane of the chick embryo, and that infected cells produce virus slowly. This latter finding is in keeping with the observation that there is a low concentration of virus in the transmissible sarcoma. Indeed, it may be essential that cancer viruses gear their rate of increase to the metabolism of the infected cell. Otherwise they would destroy the host cell instead of stimulating it to proliferate. The relatively slow rate of reproduction of the Rous virus suggests a study of double infection with viruses, as reported in an early paper. A study of the effect of a second virus upon a virus-induced tumor may be of interest.

Part IV, dealing with fowl tumors, concludes with a paper exposing differences between chemically induced and virus-induced tumors. For the purpose of this publication the absence of demonstrable virus in the chemically induced tumors has been of most interest because it has emphasized the possibility that viruses may be but one of a variety of agents capable of producing tumors. There is no need to enter into a discussion of this

point, since none of the contributors have claimed that viruses are responsible for all tumors. Only patience and work will supply answers to the etiology of all cancers.

This is an appropriate time to comment upon the investigations suggesting a tumor-inducing virus in *Drosophila*. There were several points of similarity between the insect and Rous sarcoma viruses during the process of purification. One of these was the increased activity of both viruses as they were freed of extraneous substances. One striking difference between them was the transmission of the insect virus through successive generations of hosts following its application to first-generation insects only. Thus far there is no evidence that the Rous virus is transmissible from generation to generation, as is the virus of visceral lymphomatosis in chickens. In this respect the insect virus does resemble a mammary tumor virus of mice that is transmitted through successive generations. Also, as with the insect virus, strains of mice vary in their abilities to transmit it. This brings us to the third virus-induced tumor presented for our consideration, namely, mammary cancer in mice.

Evidence that a transmissible agent was involved in the occurrence of mammary cancer in mice was reported in 1936. The virus has received considerable attention since that time, and many of its biological properties have been determined. Under natural conditions it is transmitted through generations of inbred mice by way of the mother's milk; it can be detected in the tissues of infected mice which do not develop mammary tumors until middle age. For our purpose, the biological properties of the virus are not as important as is its contribution to our knowledge of cancer viruses. In this respect, its role has been to emphasize the complexity of this virus-induced tumor.

Mammary cancer in mice had been studied for years, and hormonal factors were known to be of prime importance in their occurrence. Because the disease showed a tendency to occur in families of mice, inherited factors were also found to be of great importance. Following this lead, geneticists developed inbred strains of mice, of which some were highly susceptible to the disease while others were very resistant. While studying the relationship between the hormonal and genetic factors, they discovered the virus. Indeed, it is most difficult to understand how this virus could have been exposed until the importance of hormonal stimulation had been recognized and inbred strains of mice had been established. Perhaps we can refer to this virus as the "elusive cancer virus" in view of the fact that it was hidden for so many years behind its hormonal and genetic allies.

Years of research have slowly established the importance of the virus in the causation of breast cancer in mice. This virus produces cancer in only those mice whose genetic constitutions make them susceptible to it, and who have received adequate hormonal stimulation. There is a balance between these three factors in the sense that tumors will not arise unless a small amount of one factor is compensated by an increase in one or both of the others. For practical considerations the virus is very important in most susceptible inbred strains of mice, for when it is eliminated from the strain

the mice develop few breast tumors unless they are exposed to an abnormally large amount of hormonal stimulation. This stimulation can be supplied by forced breeding, synthetic estrogens, or transplanted pituitaries. For the purpose of this discussion, however, the fact remains that the presence of the virus is not essential for the production of breast cancer in mice. Chemical carcinogens are also able to induce these tumors in strains of mice that, under normal conditions, develop few breast tumors and do not harbor the virus.

The contribution of this virus to the aim of this monograph is twofold:

(1) The virus is not implicated in all mammary cancers of mice. This does not imply that viruses are not involved in other such cancers of mice, but it demonstrates clearly that the discovery of a viral etiology for one type of tumor by no means proves a similar etiology for all tumors of the same type.

(2) Even under natural conditions the mammary cancer virus is largely dependent upon hormonal and genetic influences in its capacity to evoke tumors. This indicates that the search for tumor viruses can be most difficult, for they may play a major or minor part in a complicated interplay of influences that eventuate in malignancy. Perhaps the complexity revealed by the mouse mammary tumor virus is what led one of the most experienced of cancer investigators represented in these pages to employ hormonal stimulation and a chemical carcinogen when he explored the possibility of the involvement of vaccinia virus in the occurrence of skin cancer in mice.

Surely the program committee selected three viruses that not only reveal the diversity of cancer viruses, but also warn investigators that new concepts may be necessary and new avenues of approach explored before we can establish the viral etiology of other tumors. All three viruses (one involved in visceral lymphomatosis, one in the Rous sarcoma of chickens, and the last in mammary cancer of mice) are infectious because they can be transmitted to new hosts. Viruses of Rous sarcoma and lymphomatosis can elicit antibodies in chickens but, thus far, the mouse virus has not proved to be antigenic in mice. The lymphomatosis virus is contagious and transmitted via saliva and feces; it is also egg-borne. The mouse virus is not readily contagious; it is transmitted mostly by way of the mother's milk but, as we have heard, it can be transmitted from males to females. The Rous virus is not contagious; at most, it is far less contagious than the other two.

The lymphomatosis and mammary tumor viruses can remain quiescent in their hosts for long periods of time, and many hosts need not develop neoplasms. The Rous virus induces tumors very rapidly. The word "quiescent" is used instead of "latent" because of the definition of latency presented in these pages by one of the contributors. Also, the implications of the constancy and indestructibility of deoxyribonucleic acid in the study of acquired tolerance, as viewed by another contributor, is easily understood by cancer workers who deal with quiescent forms of cancer viruses.

Each virus produces its characteristic type of tumor. Those tumors induced by the lymphomatosis virus are widespread, while the mouse virus induces breast tumors only. Both of these viruses induce tumors remote from the site of administration, whereas tumors always arise at the site of application of the Rous virus.

The Rous virus produces tumors in young and adult chickens. The lymphomatosis virus can also produce tumors in young and old hosts, but the young animals are more susceptible. Young mice are far more susceptible than older ones to infection with the mammary tumor virus. This age variation in susceptibility to the three prototype viruses brings us to a discussion of mouse leukemia, which has received most attention in Part V.

We have been told that only newborn mice are susceptible to the leukemia virus. This introduces a new limitation to cancer viruses. If substantiated, this observation will be of the utmost importance to cancer research, for it will introduce a new concept. Like all discoveries, however, it will also raise new problems; and the most pressing problem at present is the necessity to prove that the newborn animals do not possess a tendency to develop spontaneous leukemia.

The reason for the necessity of a thorough knowledge of the tendencies of the newborn host can be explained by using pulmonary tumors of mice as an example. Some inbred strains reveal a high incidence of spontaneous pulmonary tumors, others a low incidence. When carcinogenic compounds are injected into the susceptible mice, they develop many pulmonary tumors; these begin to appear within a few months after exposure to the carcinogen. For the purpose of this discussion, the response of resistant strains is more important for, despite the resistance of these strains to spontaneous pulmonary tumors, they do develop induced tumors following an adequate dose of carcinogen. The production of pulmonary tumors in susceptible or resistant strains is not considered evidence for the viral etiology of these tumors. This example has a significant bearing upon the reports in this publication, for it appears that not only are newborn hosts essential for transmission of the leukemia virus but that, within a strain of inbred mice, one substrain is highly susceptible. It was disturbing to hear that in one laboratory this special substrain is showing an appreciable incidence of spontaneous leukemia.

The task of evaluating the use of newborn mice as test animals for the presence of a leukemia virus will be difficult and will require much time and effort on the part of the investigators, as well as patience and understanding from those who are observers. Numerous inbred strains are available and useful in cancer research; some of these even reveal a high incidence of spontaneous leukemia. Cell-free extracts of leukemia from various strains must be administered to the newborn of a variety of strains to establish a foundation for future work. Aside from the chief objective of such investigations will be the contribution to cancer research in general of a thorough study of what can be expected when the newborn are used as test animals.

Even after the efficacy of the new host is explored, the task of determining the role of a virus in mouse leukemia will not be easy. As in fowl leukoses, there are a variety of mouse diseases that can be called a leukosis complex. Furthermore, in the mouse there are not only different types of leukosis, but also lesions that closely resemble leukosis; these may trap the unwary. The situation is more complex than the mammary tumor problem in mice, for leukemia can be induced in mice by hormonal stimulation, chemical car-

cinogens, and X irradiation, and these arise in mice belonging to low leukemic strains. It would be of much interest to know whether cell-free extracts of tumors produced in resistant strains by these techniques are capable of producing leukemia when administered to newborn mice. If not, they would serve as excellent controls for leukemias occurring spontaneously in high leukemic strains.

In case anyone does not consider these problems to be very difficult, he should add to them the additional obstacles presented in Part V. It appears that the inoculation of newborn mice with leukemic extracts produces not only leukemia, but also tumors of the parotid gland and subcutaneous sarcomas. In fact, these three tumors were presented to us as another "complex." The occurrence of parotid gland tumors and sarcomas brings us back again to the test animal. Sarcomas do occur spontaneously in the strain reported to us, and we have learned that in one laboratory the special sub-strain in which parotid gland tumors were induced has developed a few spontaneous tumors of the same type. Curiously, there are reports that even within inbred strains there is a familial tendency in the occurrence of parotid gland tumors: that is, the lesions are confined for the most part to members of the same litter. It would be of considerable interest to know if those reporting here on large numbers of these tumors have also observed a similar tendency in their experimental animals.

The interest displayed here in parotid gland tumors by those who, we hope, are primarily interested in leukemia may be justifiable if the same agent is responsible for both lesions. However, if the parotid gland tumors represent another problem, then the investigators are dividing their attentions and are thus delaying the exposure of the leukemia virus. If this should prove to be true, we could classify the leukemia virus as the "clever cancer virus" for its ability to divert attention to other tumor types.

Perhaps more rapid strides would be made in the study of the leukemia virus if its activity could be increased and if more of its biological characteristics could be ascertained in order to supply other laboratories with the virus. This is no problem with the three other viruses we have discussed. All are readily available, the Rous virus in the dried state and the others in animals or tumors that are known to contain them. We have read encouraging information about the successful transmission of the leukemia virus through successive generations; this may result in an increase in its activity. Once the virus is sufficiently active to induce the disease in a high proportion of test animals, various workers will probably become interested in it.

Attempts to culture the virus on the chorioallantoic membrane of the chick embryo could be made. Tissue-culture techniques may lead to an increase in its activity. Since newborn mice are susceptible, tissue cultures from the mouse embryo might support its multiplication. If, as claimed previously, the virus is egg-borne, then tissue cultures of the infected embryos should by all means be tested for its presence. Since hormonal stimulation, chemical carcinogens, and X irradiation are known to induce leukemia in mice these three agents could be combined with virus-containing extracts to ascertain whether an appropriate combination will elicit with certainty a

sufficient incidence of leukemia to draw the interest of other investigators to the field.

In conclusion, no one can dispute the fact that viruses are involved in the cancer process. The virus theory no longer elicits violent reactions from its opponents, and its proponents in this publication have conceded that viruses may not be involved in all forms of cancer. The theory has attained a high level of respect in cancer research, for it enables the investigator to design an intelligent approach to the many problems to be confronted. Those who pursue this course objectively are certain to contribute to the control of the disease; for it is no longer a question of whether viruses induce tumors, but in how many neoplastic processes of different species they are involved.

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